

ROLE OF LEUKOCYTE-SPECIFIC PROTEIN 1
IN ACUTE LUNG INFLAMMATION

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By
LE NGUYEN PHUONG KHANH

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Canada

OR

Dean
College of Graduate Studies and Research
University of Saskatchewan
107 Administration Place
Saskatoon, Saskatchewan, S7N 5A2
Canada

ABSTRACT

Leukocyte-specific protein 1 (LSP1), an F-actin binding protein, is involved in neutrophil recruitment into peritoneum. Because mechanisms of excessive migration of activated neutrophils into inflamed lungs, credited with tissue damage, are not fully understood, we explored the hitherto unknown expression and role of LSP1 in neutrophil migration in a mouse model of acute lung inflammation. We induced acute lung inflammation through intranasal *E. coli* lipopolysaccharide (LPS) (80µg) in wild-type 129/SVJ (WT) and LSP1 deficient (LSP1^{-/-}) mice. WT (n=10) and LSP1^{-/-} (n=11) mice showed significant neutrophilia and more neutrophils in broncho-alveolar lavage (BAL) at 9 hour post-LPS challenge compared to respective saline-treated controls (WT=7; LSP1^{-/-}=10). LPS treatment induced more BAL neutrophils (P<0.001), myeloperoxidase concentrations and Gr-1⁺ neutrophils in lung tissues in WT mice compared to LSP1^{-/-} mice. Lung myeloperoxidase and Gr-1⁺ (P<0.05) were higher in LPS-treated WT compared to the LSP1^{-/-} mice. Lung tissue and BAL fluid KC, MCP-1, MIP-1α and MIP-1β concentration and vascular permeability were not different between LPS-treated WT and LSP1^{-/-} mice but TNF-α concentration was higher in LPS-treated WT mice. Hematoxylin and eosin staining showed more septal congestion in LPS-treated WT mice compared to LSP1^{-/-} mice. LSP1 expression was increased in lungs from LPS-treated mice compared to saline control. The autopsied lungs from septic humans, compared to their respective controls, showed increased expression of LSP1. These data show that LSP1 expression is modulated in acute lung inflammation and that LSP1 deficiency reduces neutrophil migration into acute lung inflammation.

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LIST OF ABBREVIATIONS

aa:	Amino acid
AHL-12:	N-(3-oxododecanoyl)-L-homoserine lactone 12
ALI:	Acute lung inflammation
ARDS:	Acute respiratory distress syndrome
BAL:	Broncho-alveolar lavage
bp:	Base pair
BSA:	Bovine serum albumin
CINC:	Cytokine-induced neutrophil chemoattractant
<i>E. coli:</i>	<i>Escherichia coli</i>
fMLP:	Formylmethionyl-leucyl-phenylalanine
F-actin:	Filamentous actin
G-CSF:	Granulocyte colony-stimulating factor
HBSS:	Hank's balanced salt solution
HIV:	Human immunodeficiency virus
HRP:	Horseradish peroxidase
H&E:	Hematoxylin and eosin staining
HUVECs:	Human umbilical vein endothelial cells
ICAM-1:	Intercellular adhesion molecule 1
IHC:	Immunohistochemistry
IL-1 β :	Interleukin-1 β
IFN- γ :	Interferon- γ
ip.	Intraperitoneally
KC:	Keratinocyte-derived chemokine
LFA-1:	Leukocyte function-associated antigen-1
LPS:	Lipopolysaccharide
L-selectin:	Leukocyte selectin

LSP1:	Leukocyte-specific protein 1
LSP1 ^{-/-} :	LSP1 deficiency
MAPKAPK2:	Mitogen-activated protein kinase-activated protein kinase 2
MCP-1:	Monocyte chemoattractant protein 1
MIF:	Macrophage-inhibitory factor
MIP:	Macrophage inflammatory protein
MPO:	Myeloperoxidase
NETs:	Neutrophil extracellular traps
p38 MAPK:	p38 mitogen-activated protein kinase
PAF:	Platelet-activating factor
PAGE:	Polyacrylamide gel electrophoresis
PBS:	Phosphate-buffered saline
PKC:	Protein kinase C
pLSP1:	phosphorylated LSP1
PMA:	Phorbol 12-myristate 13-acetate
PMNs:	Polymorphonuclear leukocytes
pp38 MAPK:	phosphorylated p38 mitogen-activated protein kinase
PRRs:	Pattern-recognition receptors
P-selectin:	Platelet selectin
PSGL1:	P-selectin glycoprotein ligand
ROS:	Reactive oxygen species
SDF-1	Stromal cell-derived factor 1
SDS:	Sodium dodecyl sulphate
<i>S. pneumonia</i> :	<i>Streptococcus pneumonia</i>
TBS:	Tris-buffered saline
TGF-β:	Transforming growth factor-β
TLRs:	Toll-like receptors

TNF- α :	Tumor necrosis factor α
TRAF1:	TNF receptor-associated factor 1
VCAM-1:	Vascular cell adhesion molecule 1
VEGF:	Vascular endothelial growth factor
vWF:	von Willebrand factor
WT:	Wild-type 129/SvJ

1. INTRODUCTION

In 1988, lymphocyte-specific gene 1 (LSP1) encoding 300-330 amino acid phosphoprotein of 47-60 kD molecular weight was recognized in pre-B cells, B cells, and concanavalin A-stimulated murine thymocytes (1, 2). In humans, this protein, coded by the gene located at p15.5 on chromosome 11, has 67% homology to the mouse gene (3, 4). Because the protein is expressed in monocytes, macrophages, neutrophils and endothelium, the name was changed to Leukocyte specific protein 1 (5-7). It is reported that LSP1 rs3817198T>C polymorphism was associated with an increased risk of developing breast cancer (8). Furthermore, interaction of this protein with C-type lectins could allow human immunodeficiency virus (HIV) to enter dendritic cells (9). The COOH- terminal of LSP1 is responsible for the association of LSP1 with the cytoskeleton, especially filamentous-actin (F-actin), in the filopodia, lamellipodia, ruffles, and the actin-rich cell cortex of neutrophils during chemotaxis *in vitro* (2, 10, 11). LSP1 is a major downstream substrate of p38 mitogen-activated protein kinase as well as protein kinase C. Taken together, this protein appears to have important roles in cell signaling and functions (7).

Neutrophils are prominent players in acute lung inflammation, which accompanies many infectious and non-infectious lung diseases of animals and humans. For example, acute respiratory distress syndrome, one of the most serious forms of acute lung inflammation (ALI), causes 40% mortality in nearly 200,000 patients in the USA every year and results in significant healthcare costs (12, 13). The annual economic losses inflicted on animal industry in the United State of America and Canada by acute respiratory diseases of domestic animals caused by pathogens such as *Mannheimia hemolytica*, *Streptococcus pneumonia*, and *Escherichia coli* (*E. coli*) runs into billions of dollars (13-15). The most common signs of ALI include edema caused by an increase in permeability of the alveolar capillary wall, impaired blood oxygenation and exuber-

ant migration of activated neutrophils into inflamed lungs (16, 17). Nevertheless, treatment for respiratory diseases of bacterial origin is becoming challenging because of emergence of antibiotic resistant bacteria, and side effects of prolonged medication such as high doses of corticosteroids (18), adrenal insufficiency, osteoporosis, brittle skin, vision and abnormal growth (19). Because of the ongoing mortality, morbidity and economic losses associated with acute lung inflammatory diseases, there is a need to understand the molecular mechanisms to develop better therapeutics to manage respiratory diseases.

Leukocyte migration into an inflamed organ is one of the earliest signs and also a hallmark of ALI. The migration of activated leukocytes follows a molecular cascade that involves many surface adhesive proteins expressed on leukocytes and the endothelium (13). In addition to the adhesion, the leukocytes reconfigure their shape through modulation of their cytoskeleton to navigate the vascular barrier and the interstitium. There is evidence of increased polymerization of F-actin under the plasma membrane to induce shape changes in the leukocytes to facilitate their locomotion (13, 20). The activated leukocytes also release proteases and reactive oxygen species (ROS) to disrupt the barrier to migrate and also to increase vascular permeability (13, 20). It is believed that while neutrophils play an important role in host defense including in the lung, the excessive migration of activated neutrophils causes significant tissue damage resulting in mortality and morbidity (20, 21). Therefore, we need to develop new molecular interventions to fine-tune the migration of neutrophils into inflamed lungs to balance their defensive functions against their deleterious actions. However, to achieve this goal, we need to develop a deeper and more precise understanding of the molecules that regulate their migration into inflamed lungs.

There are data on the role of LSP1 in leukocytes, especially neutrophil recruitment in the vasculature of cremaster muscles, peritoneum, knee joint and skin (7, 22, 23). However, to

our knowledge, there are no such data on the expression of LSP1 in normal and inflamed lungs or on its role in the migration of leukocytes in acute inflamed lungs. Therefore, I hypothesized that LSP1 expression was altered in inflamed lungs and that LSP1^{-/-} inhibited acute lung inflammation in mice through inhibition of neutrophil migration.

2. LITERATURE REVIEW

2.1. ACUTE LUNG INFLAMMATION

2.1.1. Pernicious influences of acute lung inflammation on society

In 1967, acute respiratory distress syndrome (ARDS), one of the most serious forms of acute lung injury, was identified as a lethal condition in adult (24). In children, the incidence of ALI is significant and there is a correlation between severity of arterial hypoxemia and high mortality (25, 26). Approximately 40% of mortality that occurs in nearly 200,000 American patients every year is the result of ARDS (12, 13, 27). Additionally, the mortality of ARDS in human reaches to 34% in Australia (28) and 57.9% in Europe (29), representing significant perilous outcomes of ALI.

The annual economic losses inflicted on the animal industry in the United State of America and Canada by acute respiratory diseases of domestic animals runs into billions of dollars (13-15). The economic cost of fatal pneumonia associated with *Mannheimia haemolytica* alone in beef cattle is reported to be in the range of billion dollars in Alberta, Canada (30).

The acute lung injury manifests itself in lung edema caused by an increase in permeability of the alveolar capillary wall and impaired blood oxygenation (13). Furthermore, bronchial and alveolar epithelial cells are also damaged (13, 20). No “gold standard” for diagnosing ALI has been demonstrated until now (31). In addition to high mortality, pulmonary, neuromuscular, cognitive and psychological dysfunctions are consequences of long term lung injury. Financial burden during and after this critical condition is incalculable (31).

2.1.2. Etiology of acute lung inflammation

The most common cause of ALI and ARDS is bacterial and viral pneumonia (32, 33). In animal, many pathogens and their toxin induce acute pneumonia such as *Actinobacillus pleuropneumoniae* in pigs, *Rhodococcus equi* in foals, a Gram-negative *E. coli*, Gram-positive *Streptococcus pneumoniae* (pneumococcus, *S. pneumoniae*), severe acute respiratory syndrome (SARS) coronavirus and swine influenza virus(16, 17, 27, 34, 35). In pigs, *Actinobacillus suis*, *Streptococcus suis* and *Haemophilus suis* enter through inhalation and reside in the airways. Environmental stressors such as air pollution, high temperature and humidity cause bacterial replication. These organisms express virulence molecules such as capsular molecules, fimbriae, outer-membrane protein that injure epithelial and endothelial cells to result in fibrinogen leakage and even mortality (36).

There are data to show contributions of environmental and genetic factors to the ALI susceptibility and severity. Chronic alcohol abuse (37) and cigarette smoke exposure (38) increase the risk of developing ALI. PPFIA1 gene encoding liprin alpha, a protein involved in cell adhesion, integrin expression, and cell-matrix interactions, is considered a potential ALI risk gene (39). FAS receptor (CD95) modulating apoptosis was also reported to associate with ALI susceptibility and risk of death (40). Drugs, burns, inhalation injury and pancreatitis are concerned as potential risk factors for ALI (31).

Intra-tracheal instillation of LPS, which is derived from Gram-negative bacterial cell wall, causes ALI, and is commonly used to model bacterial ALI. The LPS treatment alters the abilities of polymorphonuclear leukocytes to transit the pulmonary vasculature because of their increased stiffness and expression of adhesion molecules (20, 41). Macrophages/monocytes

stimulated by LPS produce early pro-inflammatory cytokines such as tumor necrosis factor α (TNF- α), interleukin-1 (IL-1), IL-6 and interferon- γ (IFN- γ) to protect against infection. Moreover, these leukocytes can cause late release of pro-inflammatory cytokines, for example high mobility group box 1 (HMGB1) contributing to the tissue injury repair at the later stage (42-44).

2.1.3. Mechanisms of acute lung inflammation

The acute lung inflammatory mechanisms are incompletely understood and are dependent on the initiating causes of the lung injury. The primary mechanism of lung inflammation involves the activation of alveolar macrophages to produce cytokines after pathogen has invaded the lung. In the airway, alveolar macrophages release cytokines (IL-1, IL-6, IL-8, IL-10) and TNF- α to stimulate chemotaxis and activate neutrophils (13). Stimulated leukocytes and chemokines, a kind of cytokine, are proven to have an important function in ARDS pathogenesis (45). Moreover, there is presence of anti-inflammatory mediators including IL-1 receptor antagonist, soluble TNF receptor, auto-antibodies against IL-8, and cytokines such as IL-10 and IL-11 in the alveolar milieu (13, 20). However, an imbalance between the pro-inflammatory and anti-inflammatory cytokines is believed to occur in ALI (46).

Neutrophils are the first immune cells to migrate into inflamed organs and their migration is one of the histological hallmarks of acute lung inflammation. These leukocytes play an important role in host defense, especially in fighting bacteria, which induce lung inflammation (20, 21). Neutrophils migrate through capillary wall into the interstitium and finally into the alveoli. Activated neutrophils release cytotoxic chemicals such as proteases, ROS (47), leukotrienes, pro-inflammatory cytokines, platelet-activating factor (PAF), and procoagulant molecules, which lead to an injury of endothelium and formation of lung edema (13, 20). ROS and molecu-

lar interactions such as TLR4-TRIF-TRAF6 signaling (Toll-like receptors 4 -Toll-like receptor adaptor molecule - TNF receptor-associated factor 6) relate to important pathways in controlling ALI severity (48, 49). The migration of neutrophils can cause protein-rich hyaline membranes and protein-rich edema in the air space because of protein leakage from pulmonary vasculature into interstitium of the lung through disruption of endothelial barrier, resulting in high morbidity and mortality (50). In addition, activated platelets are believed to induce formation of neutrophil extracellular trap (NETs). NETs detected in the lungs and plasma of human transfusion-related acute lung injury (TRALI) may contribute to lung endothelial injury (51). Moreover, antimicrobial products in NETs and histones can injure endothelium and alveolar (52). The events of neutrophil migration and edema formation are regarded as cardinal pathophysiologic events in ALI (32, 53).

2.2. NEUTROPHILS

2.2.1. Neutrophil introduction

Neutrophils, polymorphonuclear granulocytes, constitute 60-75% of circulating leukocytes in most carnivores. These leukocytes were first described as cells aggregating around an injury site by Elie Metchnikoff [88]. Bone marrow produces $5-10 \times 10^{10}$ neutrophils per day to maintain equilibrium. The number of mature neutrophils in bone marrow is ten times greater than in circulatory system (54). Ten-fold increase of neutrophil quantity may occur during bacterial infection (55). The most important abilities of neutrophils are their immigration capability, killing and inhibiting microbial growth (56).

2.2.2. Neutrophil activation

Neutrophils are motile cells that are activated by pro-inflammatory cytokines, LPS, and other agents. The active stage is recognized at the inflammatory site. Neutrophil activation is also induced by pathogens or their components such as pathogen-derived formylated peptides (57). Activated neutrophils, which are recruited from the blood flow to injured tissues, are the primary component of innate immune response to pathogens. In general, after being stimulated by bacterial products such as LPS, endothelium exhibits P-selectin (CD62P) on its cell surface, which is basically a glycoprotein stored in cytoplasmic granules. P-selectin attaches to L-selectin (CD62L) on activated neutrophil surface to slow down neutrophil speed in small venules. After that, endothelium releases PAF to stimulate expression of adhesive protein named leukocyte function-associated antigen-1 (LFA-1, CD11a/CD18, integrin) on neutrophils. A firm adhesion will occur when LFA-1 of neutrophils binds an intercellular adhesion molecule-1 (ICAM-1, CD54) on endothelium. Neutrophils stopped in the blood vessels releases elastase to eliminate an anti-adhesive protein (CD43, leukosialin) on neutrophil surface for stronger binding with endothelium (58).

After being triggered by IL-1, IL-23, TNF- α , endothelium shows E-selectin adhesion molecule (CD62E), and secretes chemokine IL-8 (CXCL-8) to solicit more neutrophils (54). TNF- α also triggers the endothelium to produce IL-1 causing vasodilation, procoagulant activity, thrombosis, adhesive and chemotactic molecules. TNF- α also attracts more neutrophils. Additionally, when LFA-1 binds ICAM-1, neutrophils release CXCL1, 2, 3, 8 and cause protein leakage due to endothelial contraction and intercellular junction disruption. About one-fifth of neutrophils emigrate through endothelium, remaining leave venules through endothelial junctions. Neutrophils release protease to enter basement membrane (58).

Upon activation, neutrophils secrete pro-inflammatory cytokines/chemokines such as IL-1 α , IL-1 β , IL-1RA, TNF- α , IL-6, IL-8, IL-10, vascular endothelial growth factor (VEGF), and transforming growth factor- β (TGF- β), which can promote inflammation and augment anti-microbial function of host cells at the potential infected area (58, 59). Prostaglandins and leukotrienes were demonstrated to be synthesized by activated neutrophils in driving rheumatoid arthritis (60). Activated neutrophils also secrete superoxide anion (O²⁻) generating ROS such as hydrogen peroxide (H₂O₂), hydroxyl radical (OH⁻) and hypochlorous acid (HOCl) to kill microbes (57). Neutrophils and their cytokines are also implicated in chronic inflammation and the asthma (61) in addition to acute inflammatory responses. Inhibition of neutrophil derived cytokines can be a potentially useful therapy for inflammation (52, 61, 62).

2.2.3. Microbe clearance by neutrophils

In contrast to macrophages, neutrophils assault and eat pathogen faster but they don't have capability to prolong or repeat phagocytosis. Within 20 seconds, neutrophils grasp an opsonized particle. There are 5 main stages of phagocytosis including activation, chemotaxis, adherence, ingestion, and destruction. After being stimulated by TNF- α , CXCL8 or C5a, and through integrin ligation, neutrophils produce elastase, defensins and oxidants (58).

In innate immune response, neutrophils have CD35, named complement receptor-1 (CR1), which binds complement component C3b coating microbes. Additionally, there are many pattern-recognition receptors (PRRs) covering phagocytic cells, which are ligands on bacteria surface. Neutrophils can recognize and adhere to bacteria directly through their mannose receptors or integrins (58).

Activated neutrophils create an extracellular fiber network to catch bacteria called NETs, an early event of cell death, in the inflammatory areas. NETs are decondensed nuclear DNA strands coated by antimicrobial proteins consisting of histones and granule component, for example, from azurophilic primary granules (elastase, myeloperoxidase, cathepsin G), specific secondary granules (lactoferrin) and tertiary granules (gelatinase). Therefore, NETs have important functions as a physical defence in preventing bacterial spread and suppress virulence factors. They can kill organisms before engulfing them but have deleterious impacts on the host tissue (58, 63).

There are two ways to demolish ingested organism: by potent oxidants through “respiratory burst” procedure or involvement of lytic enzymes and antimicrobial peptides from intracellular granules of neutrophils. Another explanation for neutrophil killing microbes is the function of nicotinamide adenine dinucleotide phosphate oxidase (NOX) in pumping electron into phagocytic vacuole. The compensating ion movement will make a condition in the vacuole suitable through lowering of pH for activation of enzymes to kill and to digest organism (56).

In the lytic enzyme phagocytic pathway, neutrophil granules or lysosomes move toward and fuse with the ingested pathogen, generating phagosome to form phagolysosomes. Neutrophil microbicidal products such as lysosomal enzymes, ROS, and nitric oxide will kill organism (58, 64). Lysosomal enzymes including elastase, cathepsin G, lysozymes, proteases, acid hydrolases and myeloperoxidase are produced and aggregate in phagosomes to digest bacterial wall and destroy most microbes (58). Lactoferrin binds redoxiactive metal such as iron to minimize OH formation and deprive this nutrient of bacterial development. These products can injure host tissue concomitantly when going out extracellular environment although neutrophils have glutathione to impair oxidant toxic and moderate the damage (56).

2.3. NEUTROPHIL MIGRATION INTO INFLAMED LUNGS

2.3.1. Adhesion molecules

In fact, the mechanisms of neutrophil immigration from the circulatory system to inflammatory tissues in the lung are highly complex and poorly understood (6). Many proteins in the families of selectins, integrins and immunoglobulin superfamily play roles in neutrophil interactions with the endothelium. It is reported that selectins expressed on neutrophils lead to their rolling on the endothelium. Selectins, which refer to a group of transmembrane molecules consisting of L-selectin, E-selectin, P-selectin and P-selectin glycoprotein ligand (PSGL1), have a varied expression on cells. L-selectin is expressed on leukocytes and E-selectin expression is on inflammatory endothelium. Both inflamed endothelium and platelets express P-selectin (CD62P) whereas PSGL1 is found in leukocytes and endothelium (65, 66). Nevertheless, selectins have some functions in neutrophil recruitment in pneumonia. Some scientists demonstrated that lung inflammation induced with intravenous cobra venom factor (CVF) or LPS administration showed increased P-selectin expression on lung vascular endothelium to promote neutrophil migration (67). Furthermore, P-selectin derived from platelets possibly plays a more important role in neutrophil sequestration than that from endothelium of murine. Platelets activation is investigated as the reason behind the ICAM-1 expression in endothelium and an increase of neutrophil adhesive ability. The level of neutrophil migration, permeability, and prolonged survival decreased when the aggregation of platelets was prevented (68).

Next, integrins and chemokines mediate firm adhesion of neutrophils to activated endothelium. The integrins are membrane-linked proteins that adjust adhesion of cells by interaction with adhesion molecules such as ICAM-1, ICAM-2 and vascular cell adhesion molecule 1

(VCAM-1) (20). Some researchers illustrated that β 2-integrins generally mediated neutrophil adhesion to migrate across the pulmonary vascular endothelial barrier upon the interaction of CD11/CD18 β 2-integrins on polymorphonuclear leukocytes with their counter receptor, named ICAM-1, on the surface of vascular endothelial cells. It is concluded that preventing β 2-integrins on activated endothelium can arrest PMNs activation and migration responses (69). However, neutrophil's migration through pulmonary microvascular endothelium varies depending on pathogens. ICAM1, VCAM1 and E-selectin up-regulation did not affect neutrophil migration in the presence of LPS, *E. coli*, *S. aureus* and *S. pneumoniae*. Blocking ICAM1, adhesion molecule on endothelium, did not down-regulate neutrophil migrating to LPS, *E. coli*, *S. aureus* and *S. pneumonia* (14). Neutrophil adhesion in response to *E. coli*, *S. pneumoniae*, *S. aureus* and LPS was known to occur through β 2-integrins dependent pathway (70), (14).

In contrast, data also show that a β 2-integrin-independent pathway was observed in neutrophils migration in pneumonia induced with *S. aureus* (71) and *S. pneumonia* (70). CD18 inhibition did not significantly down-regulate neutrophil immigration to intrapulmonary and *S. aureus* clearance in rabbit model (71). In rat model, the expression of integrin subunits alpha-v and beta-3 in acute lung inflammation on neutrophils and endothelium following intratracheal challenge with *E. coli* was lower than *S. pneumonia* challenging. However, both bacteria enhance similar levels of neutrophil migration into lungs (72).

2.3.2. Chemokines/cytokines

Many chemokines, particularly IL-8, and CXCL5 (named epithelial neutrophil-activating protein 78 (ENA78)) produced by alveolar macrophages increase neutrophil infiltration (45). In rodent, CXCL1 (known as keratinocyte-derived chemokine (KC) or cytokine-

induced neutrophil chemoattractant (CINC)) and CXCL2 (known as macrophage inflammatory protein-2 (MIP-2)) are significantly associated with neutrophil migration (73). Monocyte chemoattractant protein 1 (MCP1, named as CCL2 or MCAF), a chemoattractant for monocytes, and its hematopoietic cell receptor CC chemokine receptor 2 (CCR2) can contribute to set up an efficient chemotactic gradient for neutrophils in lung inflammatory disorders (45, 74).

IL8, MCP1, IL6, GRO- α and RANTES but not TNF- α were produced in significantly higher amounts after *E. coli*, *S. aureu* and *S. pneumonia* stimulation and highest following LPS treatment compared to unstimulated control in neutrophil-endothelium interaction *in vitro*. In contrast, *E. coli* stimulates neutrophil migration 10 times higher than purified LPS at the equivalent LPS content. It is explained that intact bacteria have other constituent molecules, which activate neutrophils and the endothelium (14).

2.3.3. Configuration change

After tethering and rolling on the endothelium, the neutrophils change their configuration as a requirement to move from small pulmonary capillaries to the interstitium. Then they enter the airway via alveoli (75). Neutrophil cytoskeleton is also altered by inflammatory stimuli through activation of 7-transmembrane-spanning G-protein linked receptors (76).

Endothelial cytoskeleton, a complex network of actin microfilaments, microtubules, and intermediate filaments, contributes to shape change and transduce signals within and between endothelial cells. These components have important functions in controlling endothelial cell contractile forces and cell-matrix tethering forces (50). Actin cytoskeleton reorganization, orchestrated from extracellular signaling cascades, is an important event to allow neutrophil migration (77).

Previous studies demonstrated that ICAM-1 cross-linking activated p38 mitogen-activated protein kinase (p38MAPK) phosphorylation, which is necessary for cytoskeletal remodeling in endothelial cells. Inhibition of p38MAPK reduced neutrophil paracellular migration (78). Neutrophil adherence was also observed to induce p38MAPK phosphorylation in the central region of the endothelial cells after 2-6 minutes. p38MAPK isoforms, particularly MAPK kinase 3 and MAPK kinase 6 were identified as a requirement for cytoskeletal changes, consequently enhance neutrophil migration toward endothelial cell junctions (79).

Moreover, after intravascular inflammatory mediators triggered neutrophils *in vivo*, F-actin from the central site redistributed quickly to the sub-membrane cytoplasm periphery and to the microvilli of neutrophils. Besides that, the increase of F-actin polymerization in cytoplasm perimeter can change the biomechanical properties of neutrophils. These factors caused not only a rise in stiffness and viscosity level but also a decline in the deformable ability of neutrophils, which are probably the reasons for neutrophil sequestration throughout inflammatory development (76). The abnormal F-actin reconstitution affects neutrophil migration and extravasation from the vasculature into interstitial tissues (77).

In general, the recruitment of neutrophils into the lung is unique because of the diameter and tortuous nature of capillaries and the migration across the capillaries but not venules. The diameter of capillaries is such that neutrophils cannot flow through and need longer transit time to pass through pulmonary vasculature (75, 76, 80, 81). Because of such distinctive features and technical challenges in direct observation of neutrophil migration across the alveolar septum, the mechanisms of neutrophils recruitment in inflamed lung remain poorly understood. Therefore, there is a pressing need to develop a deeper and more precise understanding of the molecules that

regulate neutrophil migration into inflamed lungs to balance their defensive functions against their deleterious actions.

2.4. NEUTROPHIL INTEGRATION WITH OTHER FACTORS IN ACUTE LUNG INFLAMMATION

Most neutrophils have a short life-span (82). Their circulating half life in blood is around 6-8 hour while they stay in tissue about two or more days and die by apoptosis (54). Inflammatory stimulants such as oxidants trigger neutrophil apoptosis (82, 83).

After invading bacteria are killed by neutrophil products, which also damage the tissues, the damaged tissue starts the process of repair through molecules such as resolvin E1 and protectin D1, and polyunsaturated fatty acid-derived lipids produced by endothelium. These molecules stimulate more macrophage infiltration, engulfment and digestion of apoptotic neutrophils. Consequently, macrophages inhibit tissue damage from neutrophil products such as enzymes and ROS through uptake of necrosed and apoptotic neutrophils. Macrophage interacts with neutrophils through many proteins including CD31 and integrin $\alpha v \beta 3$. Macrophage endosome can also store, utilize and degrade enzymic content from neutrophil granules to restrict pathogen growth and have capability to repeat phagocytosis (58, 84, 85).

Apoptotic neutrophil can also release annexin I and other peptide derivatives, known as pro-phagocytic factors to stimulate macrophages to phagocytose them. Apoptotic cells stimulate M2 macrophage to release IL-10 and TGF- β to prepare for phagocytosis. These cytokines and PAF have functions in resolution of inflammation, wound repair, and restoration of tissue homeostasis. Additionally, apoptotic cells release lactoferrin to inhibit more granulocyte immigration (86). The neutrophil-macrophage cooperation also induces many aspect of innate immunity. Co-

operating with neutrophils, macrophages infiltrate to inflamed site to remove invaders or foreign particles from circular and respiratory system (87). Simultaneously, pathogen-associated molecular patterns (PAMPs), for instant LPS, bind Toll-like receptors (TLRs) on the surface of myeloid stem cells. These stem cells will be activated and produce more neutrophils (58).

2.5. LEUKOCYTE-SPECIFIC PROTEIN 1 (LSP1)

2.5.1. LSP1 introduction

In 1988, LSP-1 gene encoding a 300-330 amino acid phosphoprotein named lymphocyte specific protein 1 was identified by Jongstra-Bilen and colleagues. This protein was recognized in pre-B cells, B cells, concanavalin A-stimulated murine thymocytes, helper and cytotoxic T cell lines. A Ca^{2+} dependent feature of normal T cell development is related to LSP1 (1, 2, 4, 88, 89). However, T and B lymphocytes grow normally in mice lacking LSP1 (11). Because the protein was recognized in monocytes, macrophages, neutrophils and endothelium, the name was changed to leukocyte specific protein 1 in 1999. The LSP1 molecular weight varies from 47-60 kD (1). Western blots showed that the LSP1 protein was expressed as a 52 kD band with both NH₂-terminal and COOH-terminal domain in leukocytes and endothelial cells (4, 7).

This protein is a major downstream substrate of p38MAPK, mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) as well as protein kinase C (90). Inhibiting p38MAPK by SB203580 will restrict endothelial LSP1 phosphorylation compared to without p38MAPK inhibition under the similar conditions of neutrophil adherence (91). Moreover, aspirin-triggered 15-epimers of lipoxin, known as anti-inflammatory agents, can reduce p38MAPK and LSP1 activation, leads to blocking neutrophil chemotaxis, influx and other pro-inflammatory responses (92). It is reported that p38MAPK inhibition can prevent cell polarization and actin

bundle formation under PAF stimulation in neutrophils. However, MAPKKAPK-2 prevention or intracellular LSP1 neutralization solely reduces actin bundle formation. It suggests that LSP1 is a substrate in one of p38MAPK signaling pathways in managing cell motility (93).

2.5.2. LSP1 gene

In humans, LSP1, named as WP34, pp52 or leufactin, is coded by the gene located at p15.5 on chromosome 11. It has 67% homology to the mouse gene, which is located on distal chromosome 7 (3, 4), (94). Although both human and mouse genes have similar pattern of expression, human genome has some fragments corresponding with some LSP1 isoforms (4).

LSP1 transcription in leukocytes and fibroblasts is controlled by a 549 bp tissue-specific promoter. This area also contains an activated silencer gene, called *pp52* negative regulatory element (NRE), localizing to an 89 bp DNA segment between -324 and -235 bp area of promoter. However, leukocyte gene has *pp52* anti-NRE region, serving as an anti-silencer gene. This anti-silencer gene is a 33 bp segment between -383 and -350 bp area of promoter. Therefore, it allows LSP1 gene to be transcribed in leukocytes. In contrast, fibroblasts do not have the anti-silencer gene so the silencer gene is activated mostly, leading to inhibit LSP1 expression (95).

2.5.3. Binding sites of LSP1

In LSP1 sequence, the acidic N-terminal domain is for Ca^{2+} binding. In human LSP1, there is only one Ca^{2+} binding domain, which is different from two Ca^{2+} binding sites in mouse (4, 96). The association of LSP1 with the cytoskeleton, especially F-actin in mouse is mediated through basic COOH-terminal site (10) which is also the phosphorylation area (11, 90).

In 2000, Zhang and colleagues described that LSP1 interacts with F-actin through electrostatic affinity. The binding sites of LSP1 with F-actin are in three main regions of amino acids: 181-245, 246-295, and 306-339 which correspond to caldesmon I (CI), caldesmon II (CII), and two villin headpiece homologous regions (VI and VII)(97). The site within the residues 231–330 binds LSP1 tightly with F-actin (6). In 2003, Wong and colleagues confirmed that the residue from 300-330 was the binding site of LSP1 with cytoskeleton. The binding of LSP1 and F-actin can be restricted by changing the basic residues of domain corresponding to VII domain (2).

LSP1 phosphorylation induced after formylmethionyl-leucyl-phenylalanine (fMLP) treatment was identified at serine 204 and 252 in the basic C-terminal F-actin binding site in human neutrophils and corresponding position in mouse LSP1 is 195 and 243serine (11, 90). LSP1 phosphorylation residues overlap or are just behind the F-actin binding domains, the phosphorylation overlaps or occurs just after F-actin polarization. Some phosphorylated LSP1 (pLSP1) can also be found at trailing edge of neutrophils. Moreover, the affinity of LSP1 and F-actin can be influenced by phosphorylation of LSP1 since the CII domain binding area is right after the major phosphorylation site (serine 243) (11, 98).

2.5.4. LSP1 expression

There are reports that LSP1 is expressed on endothelium, monocytes, macrophages, neutrophils, B-cells, and T-cells including Thyl+ thymocytes (5-7). Early on, LSP1 mRNA or protein were not identified in granulocyte or non-differentiated HL60 cell lines(4). Later on, the 60 kD LSP1 expression was localized in HL60 and U937 cell lines with immunohistochemistry. Additionally, flow cytometry results show that LSP1 is recognized in CD19-positive circulating

B cells of blood but missing in pre-B cells and the CEM T-ALL cells (peripheral blood buffy coat of a child CEM with T acute lymphoblastic leukemia who had originally presented with lymphosarcoma) (1). There are data that LSP1 is not expressed in myeloid cells and other hematopoietic lineages such as erythrocytes (K562), mast cells, or megakaryocytes (HEL cell line) (1, 99). This protein is also not found on non-lymphoid cell lines or normal mouse tissues such as brain, lung, liver, skeletal muscle, kidney or testis (88, 100).

LSP1 protein was strongly expressed in cytoplasm of circulating blood monocytes, lymphocytes, and neutrophils. The LSP1 expression is noticed only in a few cells in the thymic cortex, splenic red pulp, lung, and liver (1). In thymus, double-immunofluorescence labeling shows that this protein is expressed at a higher level in medullary thymocytes, which are mature, compared to cortical thymocytes. This suggests that LSP1 may have an important function in the development of T-cell. In liver, LSP1 is not found in Kupffer cells by immuno-alkaline phosphatase immuno-cytochemistry. In tonsils, LSP1 is present in germinal centres, mantle zones, and interfollicular areas. LSP1 is expressed in plasma B cells, Langerhans' cells and dendritic cells in tonsillar T-cell areas but not in macrophages in the germinal centers and epithelium. By flow cytometry, LSP1 is observed in some CD3-positive T cells. In spleen, this technique also shows LSP1 in a lymphoid area but absent from the sinusoidal lining cells or red pulp macrophages. In lung, some alveolar macrophages express LSP1. It is believed that LSP1 expression is different in various stages of differentiation of macrophages (1, 100). Nevertheless, there have been limited studies on the expression of LSP1 in the lung and also specific cell types in the lung.

LSP1 was observed in the nucleus and cytoplasm of cultured mouse microvascular endothelial cells and human umbilical vein endothelial cells (HUVECs) *in vitro* with immunofluorescence (7). Biochemical techniques showed that LSP1 could spread from nucleus to cytoskele-

ton in HUVECs. By western blot, LSP1 was actually observed more strongly in the cytosol and nucleus than in the membrane and cytoskeleton. After activation by TNF- α , LSP1 levels in cytoskeleton increased but not in the nucleus and cytosol. In contrast, after leptomycin B administration, a substance inhibiting export from nucleus, level of LSP1 expression rose in nucleus but not in cytoskeleton. It suggests that LSP1 movement from nucleus to cytoskeleton has an important function in managing the cytoskeleton, which affects the cell shape (101).

Proteomic analysis on human neutrophil granules revealed LSP1 in gelatinase granules and specific granules associating actin cytoskeleton activity and phagosomes (102). Besides that, LSP1 was found to be associated with F-actin in the filopodia, lamellipodia, ruffles, and the actin-rich cell cortex of neutrophils during chemotaxis *in vitro* (2, 10, 11). This protein was also observed in many cases of cancer in the lymphatic cells, bone marrow and classical Hodgkin's disease (1).

2.5.5. LSP1 functions

LSP1 plays an important role in leukocyte chemotaxis into inflamed organs (23). In other words, neutrophil recruitment through postcapillary venules of LSP1^{-/-} mice is restricted even when activated by various cytokines (TNF- α and IL-1 β) and KC *in vivo*. In particular, endothelial LSP1 has a significant role in transendothelial migration of neutrophils. Moreover, intravital microscopy has shown a role for LSP1 in neutrophil migration induced by both KC and MIP2 in mouse cremaster muscle (22). Additionally, LSP1 has a function in histamine-induced increased permeability in postcapillary venules likely through its interactions with endothelial cytoskeleton (7).

Recent data shows that LSP1 has also functions in dome formation of endothelium *in vivo*. Whole-mount staining of cremaster muscle tissue or 2-photon microscopy showed a double-fold increase in the quantity of domes in wild-type mice compared to LSP1^{-/-} mice after TNF- α or IL-1 β administration. Moreover, transmission electron microscopy technique illustrated that endothelial domes completely enveloped neutrophils in wild-type but not in LSP1^{-/-} mice (101).

Additionally, LSP1 affected shape, flux and superoxide production of neutrophils (10). LSP1^{-/-} neutrophils cannot generate actin bundles during chemotaxis, as indicated by confocal microscopy data. After phorbol12-myristate 13-acetate (PMA) treatment, superoxide anion was produced by LSP1^{-/-} peritoneal neutrophils more than wild-type neutrophils. However, lack of LSP1 did not affect superoxide production level after KC stimulation to peritoneal neutrophils. The authors also concluded that LSP1 could reorganize the cytoskeleton of vermin neutrophil related to chemokine KC stimulation *in vitro*.

Nevertheless, an opposite function of LSP1 was explained by a β 2 integrin-dependent pathway. LSP1 modulates the Mac-1 (CD11b/CD18) function in binding to fibrinogen and ICAM-1 ligand, which regulates neutrophil chemotaxis. LSP1 deficiency causes neutrophil adhesion, polarization, immigration, and fibrosis in inflammation in skin and knee joints. These resulted in accelerated injury healing (23, 103, 104).

Endothelial LSP1 was phosphorylated when neutrophils adhered to the endothelium and that phosphorylation allowed neutrophil to adhere stronger. Neutrophil adhesion and endothelial LSP1 phosphorylation depended on neutrophil β 2 integrins/CD18 and endothelial ICAM-1. β 2 integrin blockage reduced lung, heart, and murine SVEC4-10EE2 endothelial phosphoryla-

tion and MIP2-stimulated neutrophil adhesion. Results were the same when ICAM1 was blocked before endothelium was administered by TNF- α . Phosphorylated endothelial LSP1 was increased by cross-linking ICAM1 or ICAM1-mediated adhesion (91). These data show that LSP1 cooperates with beta2-integrins and ICAM-1 in neutrophil adhesion to the endothelium.

LSP1 is involved in increasing motility, microfilamentous cytoskeletal abnormalities, and imperfection in actin polymerization dynamic in neutrophil actin dysfunction (NAD), observed in human immunodeficiency syndrome (105). Interaction of LSP1 with C-type lectins can also allow HIV to enter dendritic cells (9). In 2009, Anand et al. reported that siRNA LSP1 down-regulated dendritic cell migration by 50% compared to non-target siRNA treated dendritic cells and LSP1 redistributed in cytoplasmic projections and associated obviously with actin. Normal dendritic cells can inhibit HIV-1 transfer to lymphoid T cells compared with siRNAs treated LSP1 deficient dendritic cells in human. Similarly, LSP1^{-/-} bone marrow dendritic cells also enhanced HIV-1 transfection to human T-cell line. It is indicated that LSP1 regulated dendritic cell migration in response to HIV-1 gp120 (106). It is reported that different hairy cell leukemia morphology can be associated with LSP1 expression (107). LSP1 may also have a role on oncogenesis (1). In fact, results by meta-analysis showed that LSP1 rs3817198T>C polymorphism was associated with an increased risk of developing breast cancer (8).

It was known that CD64 (Fc γ RIa), a membrane glycoprotein, binds strongly with IgG antibody then interacts with γ chain having immuno receptor tyrosine-based activation motif to stimulate cells (108). This receptor can be found on the surface of monocytes, macrophages, and activated neutrophils. CD64 enhances phagocytosis, endocytosis, and inflammatory production (109, 110). Some scientists used mutant form of Fc γ RIa lacking the cytoplasmic domain tail in murine macrophages to examine the relationship of this receptor in modulation of some gene ex-

pression including LSP1. After macrophages were incubated with anti-FcγRIαAbF (ab') to achieve receptor-specific cross-linking, LSP1 gene and mRNA expression level was greater in macrophage transfected with cDNA-encoding human FcγRIα (CD64) than mutant macrophages. It was indicated that LSP1 is involved cytoplasmic domain of FcγRI α (CD64) α-chain modulating the transcriptional activity induced by receptor-specific engagement in macrophages. In other words, LSP1 is probably related to some macrophage functions consisting of phagocytosis, endocytosis, and intracellular trafficking of receptor-Ag complexes (111).

LSP1 can induce anti-IgM mediated apoptosis by regulating a Ca^{2+} dependent pathway in WEHI-231 cell and normal immature B cells. Apoptosis increase can be a result of membrane immunoglobulin M regulated by LSP1 through residue 179 and 330 (112). It was explained that this protein can bind PKC-beta I and inhibit anti-IgM-induced PKC-beta I translocation (113).

However, Laurent Sabbagh and colleagues reported that LSP1 was involved in T cell survival through 4-1BB signaling, a type 2 trans-membrane glycoprotein belonging to the TNF death receptor. Ligand 4-1BB, named CD137, which binds to TNF receptor-associated factor 1 (TRAF1), is important for CD8 T cell survival. It is suggested that LSP1 is a requirement downstream of 4-1BB to regulate proapoptotic Bcl-2 interacting mediator of cell death (Bim) content. LSP1 binds N-domain of TRAF1 of CD8+ T cells. LSP1-TRAF1 cooperation also activates extracellular-signal-regulated kinases (ERK) pathway to increase survival of T cells (114).

Taken together, LSP1 plays an important role in neutrophil chemotaxis, recruitment into inflammatory organs. To our knowledge, while significant data are available on the role of LSP1 in leukocytes, especially neutrophil recruitment in cremaster muscles, peritoneum, knee joint and skin (7, 22, 23), there are very few data on the expression and function of LSP1 in acute

lung inflammation. Therefore, I proposed a series of studies to understand the expression and function of LSP1 in a mouse model of LPS-induced acute lung inflammation.

3. HYPOTHESES and RATIONALE

It is reported that alveolar macrophages depending on the stage of differential express LSP1 (1). Nevertheless, there have been limited studies on the expression of LSP1 in the lungs. Because of the role of inter-cellular communication in organ physiology, it is important to understand cell-specific expression of a protein to elucidate its functions. Therefore, I hypothesized that (1) LSP1 was expressed in mouse and human lungs and (2) its expression was increased in inflamed lungs. (3) Additionally, LSP1 had an important role in neutrophil recruitment during acute lung inflammation.

Moreover, the role of LSP1 in neutrophils chemotaxis and migration has been examined in organs other than the lung (23). Neutrophil recruitment through postcapillary venules of LSP1^{-/-} mice is attenuated even when activated by various cytokines (TNF- α and IL-1 β) and KC in cremaster muscle (22). LSP1 also has a function in dome formulation of endothelium, a process that is important in neutrophil migration into the inflamed organs (7, 101, 115). Because the role of LSP1 in acute lung inflammation especially the neutrophil migration has not been examined, I hypothesized that LSP1 deficiency will inhibit acute lung inflammation in mice through inhibition of neutrophil migration.

4. OBJECTIVES

The role of LSP1 in acute lung inflammation has not been studied. Therefore, in this study, I proposed to investigate LSP1 expression in normal and acute inflammation lung of human and mice. Another purpose is to examine the role of LSP1 in LPS-induced acute lung inflammation in mouse model.

5. RESEARCH QUESTIONS

- Is the expression of LSP1 altered in inflamed lungs compared the normal lungs?
- Does LSP1 have a function in neutrophil migration, microvascular permeability and pathology of LPS-induced acute lung inflammation in mouse model?

6. MATERIALS AND METHODS

6.1. Materials

Twelve to sixteen-week-old male wild-type and LSP1 deficiency ($LSP1^{-/-}$) mice strain 129/SVJ were obtained from Dr. Jenny Jongstra-Bilen at University of Toronto and transferred to the Department of Pharmacology, College of Medicine, University of Saskatchewan. Autopsied normal and septic human lung (n= 5 each group) were obtained from the Department of Pathology in the College of Medicine, University of Saskatchewan. Vetalar (ketamine hydrochloride injection U.S.P, Bioniche company, Canada), Rompun (xylazine, Bayer company, Canada), lipopolysaccharide from *E. coli* 055:B5 (Sigma Aldrich, Saint. Louis, MO, USA), LSP1 antibody rabbit anti mouse, reactive in mouse and human (Novus biological, Oakville, ON, Canada), purified rat anti mouse Ly-6G Ly-6C antibody (Gr-1) (BD Biosciences Pharminogen™, Mississauga, ON, Canada), secondary antibody Polyclonal goat anti-rabbit immunoglobulins/HRP (horse-radish peroxidase), secondary antibody polyclonal rabbit anti-ratimmunoglobulins/HRP, primary antibody polyclonal rabbit anti human von Willebrand factor (vWF) (Dako, Burlington, ON, Canada), Vector® VIP peroxidase substrate kit for peroxidase (Vector laboratories, Burlingame, CA, USA), myeloperoxidase standard from human leukocytes (Sigma Aldrich, Saint. Louis, MO, USA), Bio-Plex Pro assays kit (Bio-Rad, Meyerside Drive Mississauga, ON, Canada) were purchased commercially.

6.2. Mouse model of acute lung injury

Twelve to sixteen-week-old male wild-type 129/SVJ and $LSP1^{-/-}$ mice were used. $LSP1^{-/-}$ mice were generated as described by Dr. Jongstra-Bilen and colleagues previously (91, 99). The experiments were approved by the University of Saskatchewan's Animal Research Eth-

ics Board and adhered to the Canadian Council on Animal Care guidelines. All mice were housed in 12-hour dark/light cycle and fed standard laboratory diet in Animal Care Unit, Western College of Veterinary Medicine, University of Saskatchewan and were allowed to acclimatize for one week before treatment.

Lipopolysaccharide-induced acute lung inflammation mouse model were designed as described previously (116, 117). Briefly, 80 µg of LPS in 80µl saline was instilled intranasally for each mouse to induce acute lung inflammation in wild-type (WT; n=10) and LSP1^{-/-} (LSP1^{-/-}; n=11) mice. Saline 0.9% (80µl/mouse) was used as a sham for negative control groups (7 WT and 10 LSP1^{-/-} mice). After nine hours of the treatment, all of mice were injected 100mg/kg ketamine and 10mg/kg xylazine intraperitoneally (ip.) to euthanize. Following this, cardiac puncture blood, lungs and bronchoalveolar lavage (BAL) fluid were collected. We have also collected mouse lungs from LPS intranasally treated mice after 12 and 24 hour to examine the expression of LSP1 (n=5 each).

Table 6.1. The mouse experimental design

Phenotype of mice	Number (N= 38)	Intranasal treatment
Wild-type 129/SvJ	n = 7	80 µl sterile sodium chloride 0.9%
Wild-type 129/SvJ	n = 10	80 µl LPS from <i>E. coli</i> 055:B5(1 µg/µl)
LSP1 ^{-/-}	n = 10	80 µl sterile sodium chloride 0.9%
LSP1 ^{-/-}	n = 11	80 µl LPS from <i>E. coli</i> 055:B5 (1 µg/µl)

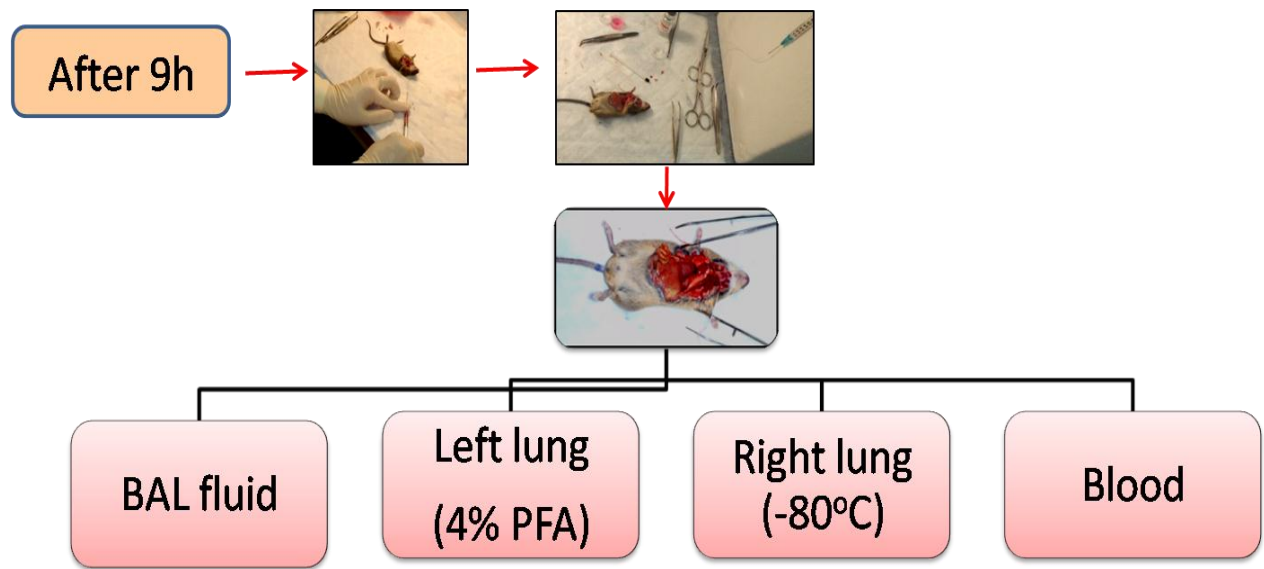


Figure 6.1. Schematic summary of acute lung injury mouse model

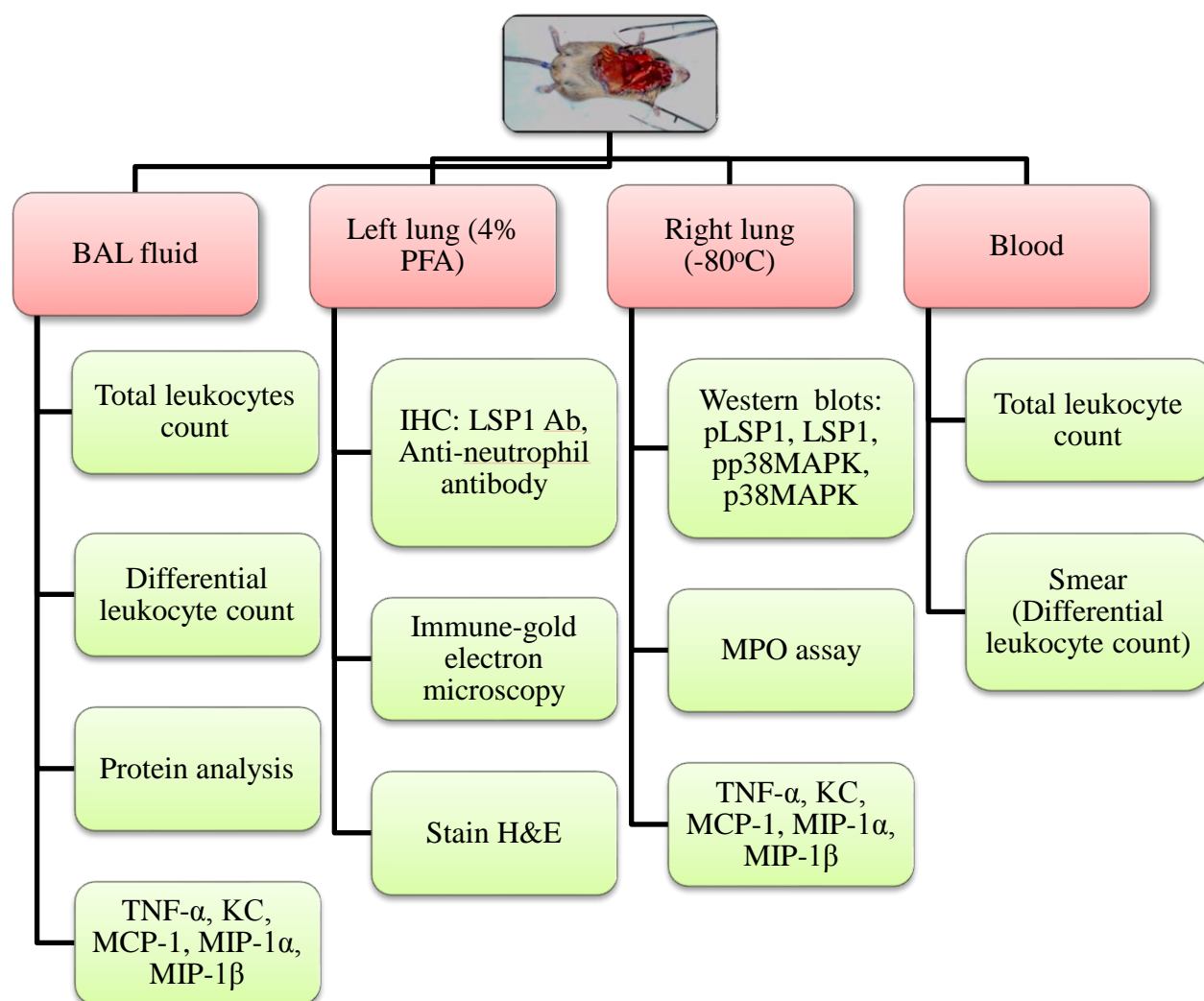


Figure 6.2. Schematic summary of sample collection and target in mouse experiment

6.3. Investigate LSP1 expression in normal and acute inflamed lungs of human and mice

6.3.1. LSP1 immunohistochemistry

LSP1 expression in mouse and human lung sections was examined by immunohistochemistry using an LSP1 antibody (Novus biological, Oakville, ON, Canada). The immunohistochemistry protocol was adjusted from a previous paper (118). Briefly, the sections were deparaffinized followed by quenching of endogenous peroxidase activity and were treated with pepsin. Following 1 hour blocking non-specific binding with 1% bovine serum albumin (BSA), the sections were treated primary antibody (20 µg/ml LSP1 antibody) and appropriate secondary antibody (1.5 µg/ml polyclonal goat anti-rabbit immunoglobulins/HRP). The color development was carried out with Vector® VIP peroxidase substrate kit. While some sections were stained with methyl green, the others were examined without nuclear staining to assess whether LSP1 is present in the nucleus. The controls included staining with the anti-endothelial marker, vWF, serving as a positive control, or IgG isotype antibody matching control instead of LSP1 primary antibody or omission of both primary and secondary antibodies serving as negative controls. Also, lungs from LSP1^{-/-} mice stained with LSP1 antibody acted as an important negative control for the non-specific staining by the antibody.

6.3.2. Western blot analyses for pLSP1 and LSP1

Frozen mouse lungs were homogenized in T-PER tissue protein extraction reagent, protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, USA.) as described previously (119). The Western blot procedure was modified from a previous protocol (72). Briefly, lysate was mixed with equal volume of 2X Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH approximate 6.8) (Sig-

ma Aldrich, Saint. Louis, MO, USA) before being loaded on 12% SDS-PAGE gel. After that, separated proteins in the gel were transferred onto HybondTM-ECL nitrocellulose membrane (GE healthcare, Germany). Next, 5% bovine serum albumin in Tris-buffered saline (TBS) Tween 0.1% was used to block non-specific binding.

To detect total LSP1 (n = 6 each group), the membranes were then incubated with polyclonal LSP1 antibody rabbit anti mouse (1:500, Novus biological, Oakville, ON, Canada) and secondary antibody polyclonal goat anti-rabbit immunoglobulins/HRP (1:2000, Dako, Burlington, ON, Canada). Incubation without primary antibody was used as a negative control. Also, lung lysates from LSP1^{-/-} mice incubated with LSP1 antibody acted as an important negative control for the non-specific binding by the LSP1 antibody. The detection blots were performed with Amersham ECL western blotting detection reagents Kit (GE Healthcare, Germany). Finally, the membranes were exposed under autoradiography Hyperfilm ECL (GE Healthcare, Germany).

For pLSP1 detection, the probed membrane was stripped with blot restore membrane rejuvenation kit (Millipore, MA, USA.) prior to be pre-probed with phosphorylated LSP1 p-ser 252 antibody (1:500, Novus biological, Oakville, ON, Canada) and secondary antibody polyclonal goat anti-rabbit immunoglobulins/HRP as above.

Finally, all membranes were stripped again and incubated with affinity purified rabbit anti β -actin antibody loading control (1:5000, abcam, Cambridge, MA., USA.) and secondary antibody polyclonal goat anti-rabbit immunoglobulins/HRP. β -actin, an abundant house-keeping gene's product expressed in all cells, was used to examine the equal total amount of protein loaded of each sample. Densitometry quantification was performed using ImageJ software to evaluate relative density of pLSP1, LSP1 expression level.

6.3.3. Human neutrophil isolation

Human neutrophils were isolated following the previously published protocols (120). Briefly, intravenous human blood was donated from healthy volunteers. Neutrophils were isolated based on density gradient principle. Histopaque H1119, H1083 and H1077 (Sigma Aldrich, Saint. Louis, MO, USA), adjusted to a density of 1.119 g/ml, 1.083 g/ml and 1.077 g/ml, respectively were carefully overlaid from the bottom to the top in order and blood was placed in the uppermost. After being centrifuged at 700g for 10 minutes in 4°C, neutrophils were aspirated in the layer between H1083 and H1119. The collection was centrifuged at 700g for 10 minutes in 4°C to collect neutrophils in pellet. Erythrocyte contaminants were lysed by distilled water isotonicity and neutrophils were restored by NaCl in phosphate buffer, pH=7.2. Then neutrophils were washed twice in 1X phosphate buffer saline (PBS), pH = 7.2 before being resuspended in 1 ml RPMI 1640 medium (Lonza company, USA).

Trypan blue was used to assess live cell quantity. Total number of viable cell collected = average number of viable cell/ 1mm^2 in haemocytometer $\times 10^4 \times$ dilution factor with trypan blue $\times 1\text{ml}$. Next, the solution was diluted to reach final concentration at 10^6 cells/ml. After that 100 μl of cells were cytopun into slide and were stained with a Hemacolor stain set (EMD Chemicals, Gibbstown, NJ) for differential leukocyte count in 10 fields at 400 \times magnification. The isolated neutrophils were used when purity was 95% or more.

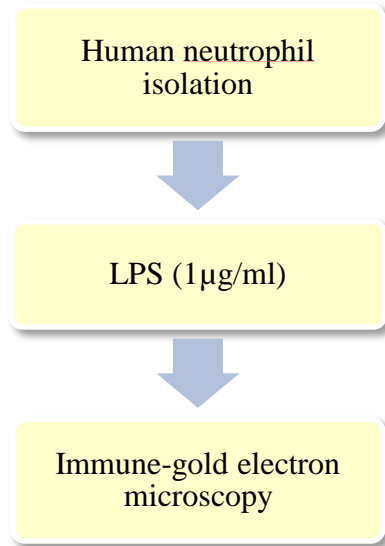


Figure 6.3. Human neutrophil experimental design

6.3.4. Immune gold electron microscopy

Human neutrophils and mouse lungs were assessed for the expression of LSP1. The isolated neutrophils treated with LPS, and mouse lungs were fixed in 2% paraformaldehyde with 0.1% glutaraldehyde in 0.1M sodium cacodylate buffer for 3 hours at 4°C. After being dehydrated, the neutrophils were infiltrated with white resin. Next, samples were sectioned at a thickness of 100nm and placed on nickel grids. Non-specific bindings were blocked by BSA 1% in Tris buffered saline before one hour incubation in LSP1 antibody (40µg/ml). Negative control without LSP1 antibody also was examined. After 3 washes in Tris buffer saline, the sections were incubated for 1 hour in 20nm gold-conjugated anti rabbit secondary antibody with 1:100 dilution factors. Following that, samples were incubated in 2% aqueous uranyl acetate, an indicator for negative staining then Reynold's lead citrate to enhance the electron-scattering properties of biological components inside the cells. The micrographs were imaged using EM410 electron microscope (Philips company, Holland) operated at 60 KV connecting a 622 F/O TV display (Gatan, Pleasanton, CA 94566, USA.).

6.4. Examine the role of LSP1 in LPS-induced acute lung inflammation in mouse model

6.4.1. Histopathological analysis

Right bronchus of mouse lung was ligated with a thread before left lung was instilled 1 ml of cold 4% paraformaldehyde *in situ* through trachea. After left lung was inflated, right lung was cut and stored at -80°C for analysis later on. Left lung was fixed in 4% paraformaldehyde, processed and embedded in paraffin. All sections (5µm thickness) were prepared and placed on coated glass slides. Sections were stained with hematoxylin and eosin for histopathological examination. Histopathology scoring was adjusted from previous description (117).

Table 6.2. Key for histopathology scoring

Grade	Lung inflammatory degree
0	Normal architecture.
1	Diffuse reaction in alveolar walls, congestion 1-10 neutrophils/field in peribronchial vascular space (magnification 1000×).
2	11-20 neutrophils/field, congestion, slight thickening alveolar wall
3	21-30 neutrophils/field, congestion, thickening alveolar wall, light epithelial damage
4	≥31 neutrophils/field, congestion, thickening alveolar wall with more than 10% of lung consolidated, epithelial damage.

6.4.2. Blood and BAL cell counts

Briefly, after trachea was exposed, lungs were lavaged three times with 0.5 ml of cold Hank's balanced salt solution (HBSS) with NaHCO₃(Sigma, UK) and the BAL was centrifuged

at 1,500g for 10 minutes at 4°C. Supernatants were collected and stored at -80°C for protein, chemokine and cytokine assay later on. Total leukocytes in BAL fluid were counted by using hemocytometer. Cells were resuspended at 10^6 /ml with HBSS. 100µl of these solutions were cytopun onto microscope slides and stained with Wright-Giemsa Hemacolor stain set (EMD Chemicals, Gibbstown, NJ) for differential leukocyte count (neutrophils, basophils, eosinophils, monocytes and lymphocytes) in 4 random fields at 400× magnification each mouse.

Mouse peripheral blood collected through cardiac puncture was kept in heparinized tubes. The total number of leukocytes/ml of blood was counted with a haemocytometer after erythrocyte hemolysis with 2% acetic acid. Simultaneously, blood smear was stained with Wright-Giemsa method (EMD Chemicals, Gibbstown, NJ) for differential leukocyte count in 10 fields at 400× magnification.

6.4.3. Lung myeloperoxidase (MPO) quantification

MPO assay was followed as previous protocol (121). Shortly, the lung homogenates in 50mM HEPES (Invitrogen Life technologies, Burlington, ON, Canada) were centrifuged at 900g for 20 minutes at 4°C and the pellet was resuspended and rehomogenised in 0.5% cetyltrimethyl ammonium chloride (CTAC) solution. MPO colorimetric assay was performed using 3, 3', 5, 5'-tetramethylbenzidine (TMB) as substrate for H₂O₂ under low pH conditions. Reaction was stopped by 1M H₂SO₄ and read at 450nm OD by NOVOSTAR software, Bio-Rad machine. Results were expressed as units of MPO per mg of lung tissue.

6.4.4. Gr-1 immunohistochemistry staining

The mouse lung sections were also stained with Gr-1 anti-neutrophil antibody to determine neutrophil migration into perivascular and peribronchial compartments. The immunohistochemistry protocol was adjusted from a previous paper (118). Briefly sections were deparaffinized followed by quenching of endogenous peroxidase activity and were treated with pepsin. Following 1 hour blocking non-specific binding with 1% bovine serum albumin (BSA), the sections were treated with primary antibody (0.40 µg/ml Gr-1 antibody) and appropriate secondary antibody (13 µg/ml polyclonal rabbit anti-rat immunoglobulins/HRP). The color development was carried out with Vector® VIP peroxidase substrate kit. The controls included staining with the anti-endothelial marker, vWF, serving as a positive control, or rat IgG_{2b}, κ antibody isotype matching control instead of Gr-1 primary antibody or omission of both primary and secondary antibodies serving as negative controls. The degree of neutrophil infiltration was expressed as a Gr-1⁺ staining score in 4 randomly chosen fields per mouse in magnification 1000× (n=5), modified method from a previous paper (122).

Table 6.3. Gr-1⁺ neutrophil scoring system

Score	Number of neutrophils/field (Magnification 1000×)
0	0
1	1-10
2	11-20
3	21-30
4	≥ 31

6.4.5. Cytokine and chemokine analyses in BAL fluid and lung homogenates

We quantified KC, MCP-1, MIP-1 α , MIP-1 β and TNF- α in right lungs and BAL fluid collected from the mice with Bio-Plex Pro Assay Kit (BioRad, Meyerside Drive, Mississauga, ON, Canada). Briefly, the lungs were ground in lysing solution, sonicated and centrifuged for the collection of supernatant. The cytokine and chemokines were quantified in 50 μ l following manufacturer's instructions.

6.4.6. Pulmonary microvascular permeability assay

Protein analysis in BAL fluid was carried out to evaluate vascular permeability (BioRad, Protein Assay Dye Reagent Concentration, USA). The principle of protein determination was according to method of Bradford (123). Briefly, 10 μ l of each standard or samples was pipetted into separate wells of 96-wells microtiter plates. After addition of 200 μ l of dye reagent and 5 minute-incubation, the absorbance was measured at 595nm by NOVOSTAR software, Bio-Rad machine.

6.4.7. Western blot analyses for pp38MAPK and p38MAPK

Frozen mouse lungs were homogenized in T-PER Tissue Protein Extraction Reagent, protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, USA) as described in a paper (119). The Western blot procedure was modified from a previous protocol (72) as described above.

To detect total and phosphorylated p38 mitogen-activated protein kinase (pp38MAPK) (n=4 each group), membranes were incubated with purified monoclonal rabbit anti phospho-p38MAPK (Thr180/Tyr182) antibody (1:1,000, Cell signaling technology, Danvers, MA 01923,

USA.) at 4°C with gentle shaking overnight and secondary antibody polyclonal goat anti-rabbit immunoglobulins/HRP for 1 hour (1:2000, Dako, Burlington, ON, Canada). Following that, the probed membranes were stripped with blot restore membrane rejuvenation kit prior to be probed with purified rabbit anti p38MAPK antibody (1:10,000, Enzo Life Sciences, Farmingdale, NY 11735, USA.) and secondary antibody polyclonal goat anti-rabbit immunoglobulins/HRP as above.

Finally, all membranes were stripped again and incubated with immunogen affinity purified rabbit anti β -actin antibody loading control (1:5000, abcam, Cambridge, MA., USA.) and secondary antibody polyclonal goat anti-rabbit immunoglobulins/HRP. β -actin, an abundant house-keeping gene's product expressed in all cells, was used to examine the equal total amount of protein loaded of each sample. Incubation without primary antibody was used as a negative control. Densitometry quantification was performed using ImageJ software to evaluate relative density of pp38MAPK, p38MAPK expression level.

6.4.8. Statistical analysis:

Statistical analysis was performed using GraphPad Prism software version 5.04 (San Diego, CA, USA). Quantitative results were expressed as mean and error bars represented standard error. Normal distribution of residuals was tested by histogram and Shapiro-Wilk test. Data were analyzed by Analysis Of Variance (ANOVA) followed by Bonferroni multiple comparison test. Student *t test* or Wilcoxon Signed Rank Test was used to compare two groups. The critical value of α was set to 0.05 as a significant difference (two-tailed).

7. RESULTS

7.1. LSP1 expression in normal and acute inflamed lungs of human and mice

7.1.1. LSP1 expression is increased in septic human lungs

Because to our knowledge there is no information on LSP1 expression in normal human lungs, we used immunohistochemistry to bridge this gap. Autopsied normal human lungs showed LSP1 on alveolar septum, alveolar macrophages, and blood cells (Figure 7.1C, E). Septic lungs showed massive migration of neutrophils and obliteration of normal lung architecture. Neutrophils in the alveoli and blood vessels showed robust expression of LSP1 (Figure 7.1D, F). The semi-quantification of immunohistochemical LSP1 staining showed significant increase in staining of LSP1 in all of the septic human lungs compared to the normal human lung controls (Figure 7.2). Interestingly, LSP1 was extensively localized into the nuclei of neutrophils. The LSP1 expression was more intense on nucleus than on cytoplasm.

Histopathology on hematoxyline and eosin-staining was also done to confirm presence of inflammatory signs of septic human lungs. Healthy human lungs showed normal histologic structure with thin alveolar septa along with intact septal cells and clear alveolar spaces (Figure 7.3A). Septic human lungs showed exuberant migration of inflammatory cells, especially neutrophils in alveolar and peribronchial vascular spaces and obliteration of lung architecture (Figure 7.3B).

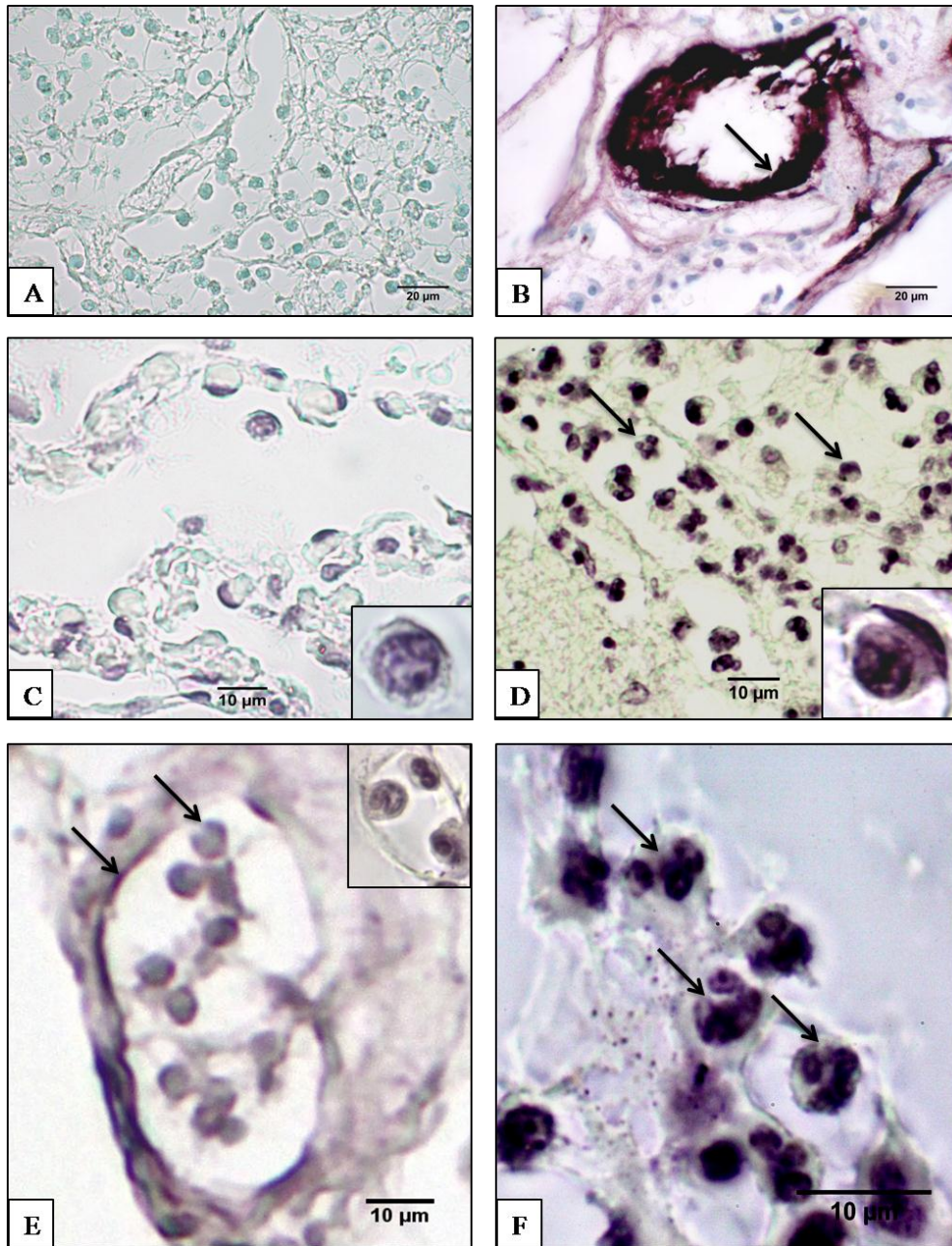


Figure 7.1. Human lung immunohistochemistry for LSP1

Lung section from humans was stained with IgG isotype antibody matched control and secondary antibody (A) shows only green blue color of methyl green counter staining serving as a negative control. Figure (B) depicts positive staining on only endothelium of lung section stained with vWF serving as a positive control. LSP1 staining (arrows) is noticed in alveolar septum and alveolar macrophage (C) and blood cells (E) in lungs from a non-septic human. Figure D and F show increased number of neutrophils expressing LSP1 (arrows) recruited into a septic lung. The LSP1 staining is mostly nuclear (F). Original magnification 400 \times , 1000 \times . N=5 in each group

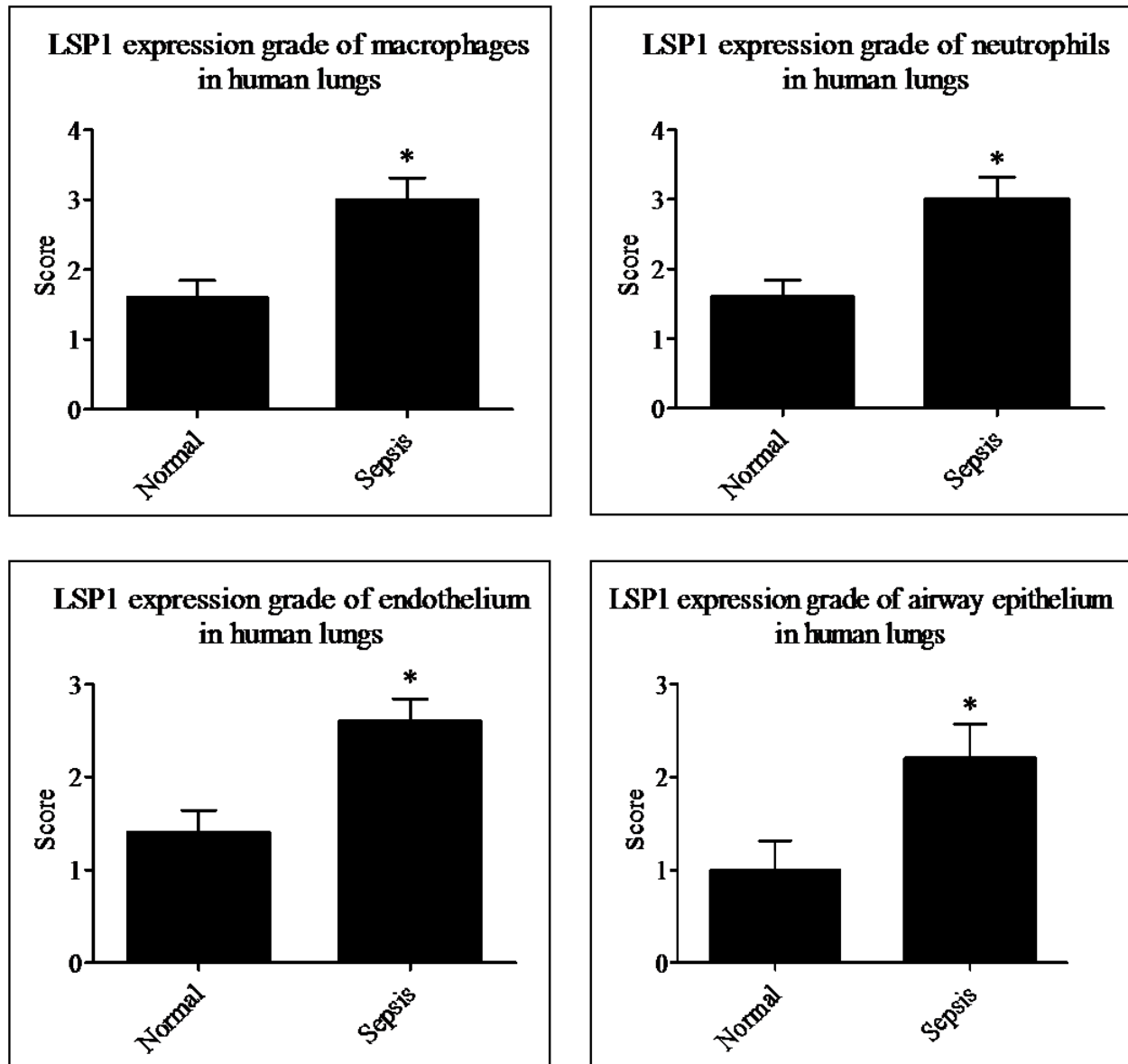


Figure 7.2. Statistical analysis semi-quantitative LSP1 expression scoring of human lung sections.

In general, LSP1 expression increases in macrophage, neutrophils, endothelium and airway epithelium of septic human lungs comparing to normal human lung control. We classified that: 0: no staining, 1+: light staining, 2+: moderate staining, 3+: strong staining, 4+: very strong staining. Asterisk (*) indicates significant difference from normal lung group ($P < 0.05$). $n = 5$ each group.

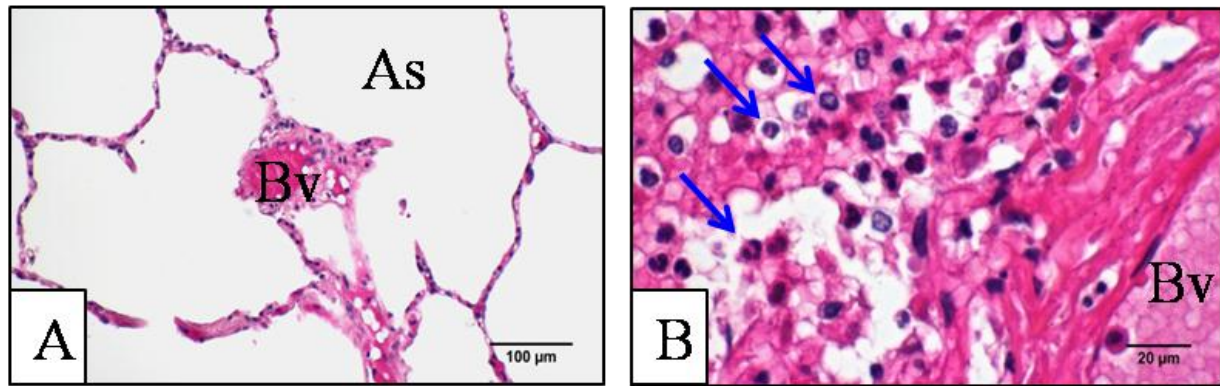


Figure 7.3. Histopathological examination of human lungs stained with hematoxylin and eosin

Healthy human lung (A) showed normal histologic structure with thin alveolar septa along with intact septal cells and clear alveolar spaces. Septic human lung (B) showed massive migration of inflammatory cells, especially neutrophils in alveolar and peribronchial vascular spaces and obliteration of lung architecture. Original magnification 100 \times (A) and 400 \times (B). (As: Alveolar space, Bv: Blood vessel)

7.1.2. LSP1 and phosphorylated LSP1 expression is increased in LPS-induced lung inflammation in mouse

After examining the LSP1 expression in human lungs, we explored LSP1 expression in a mouse model of LPS-induced lung inflammation, and compared it to the expression in lungs from normal mice. In contrast to saline-treated mice lungs, the inflamed lungs showed increased staining of LSP1 on alveolar septa, airway epithelium, and vascular endothelium at 9 hour post-LPS treatment (Figure 7.5). In particular, the staining was highly intense in neutrophils, macrophages and airway epithelium (Figure 7.5). The expression of LSP1 remained intense in various lung cells 12 and 24 hours post-LPS treatment (Figure 7.6). The LSP1 was localized mostly in nuclei of airway epithelial cells, macrophages and neutrophils at 24 hours post-LPS treatment. The semi-quantification of immunohistochemical LSP-1 staining showed significant increase in staining of LSP1 in all of the LPS-treated mouse lungs compared to the saline-treated controls (Figure 7.7). Immuno-electron microscopy confirmed the presence of LSP1 in endothelial cells and neutrophils in the lungs of LPS-treated mouse (Figure 7.8)

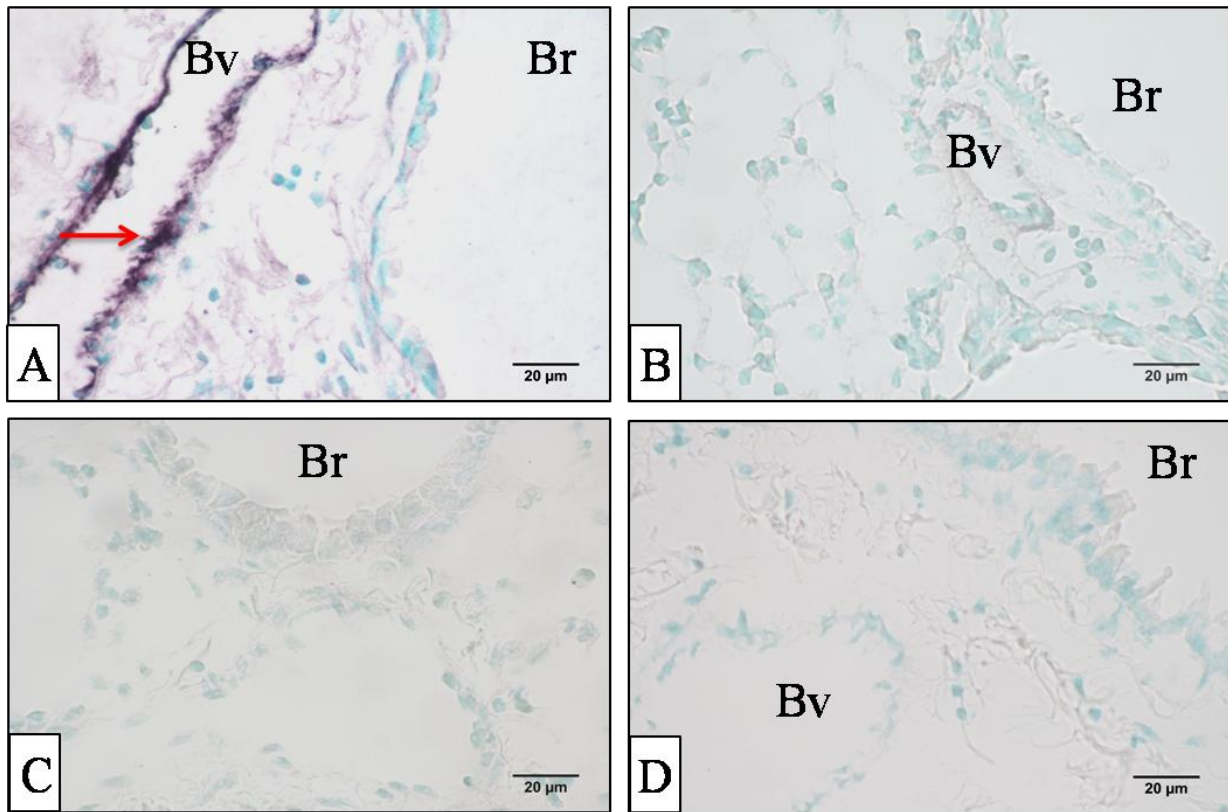


Figure 7.4. Controls for LSP1 immunohistochemistry staining of mouse lungs

Mouse lung section stained with vWF antibody (A) shows only positive reaction in blood vessel (arrow), serving as a control for the protocol. Figure (B) shows wild-type mouse lung section stained of with IgG isotype matched control instead of LSP1 antibody. Lungs from saline treated $LSP1^{-/-}$ mouse (C) and LPS treated $LSP1^{-/-}$ mouse (D) were stained with LSP1 antibody show no positive staining. Figure B, C and D serve as negative controls, which rules out non-specific binding of primary and secondary antibodies. Abbreviation: Br, bronchiolar epithelium; Bv. Blood vessel. Original magnification 400 \times .

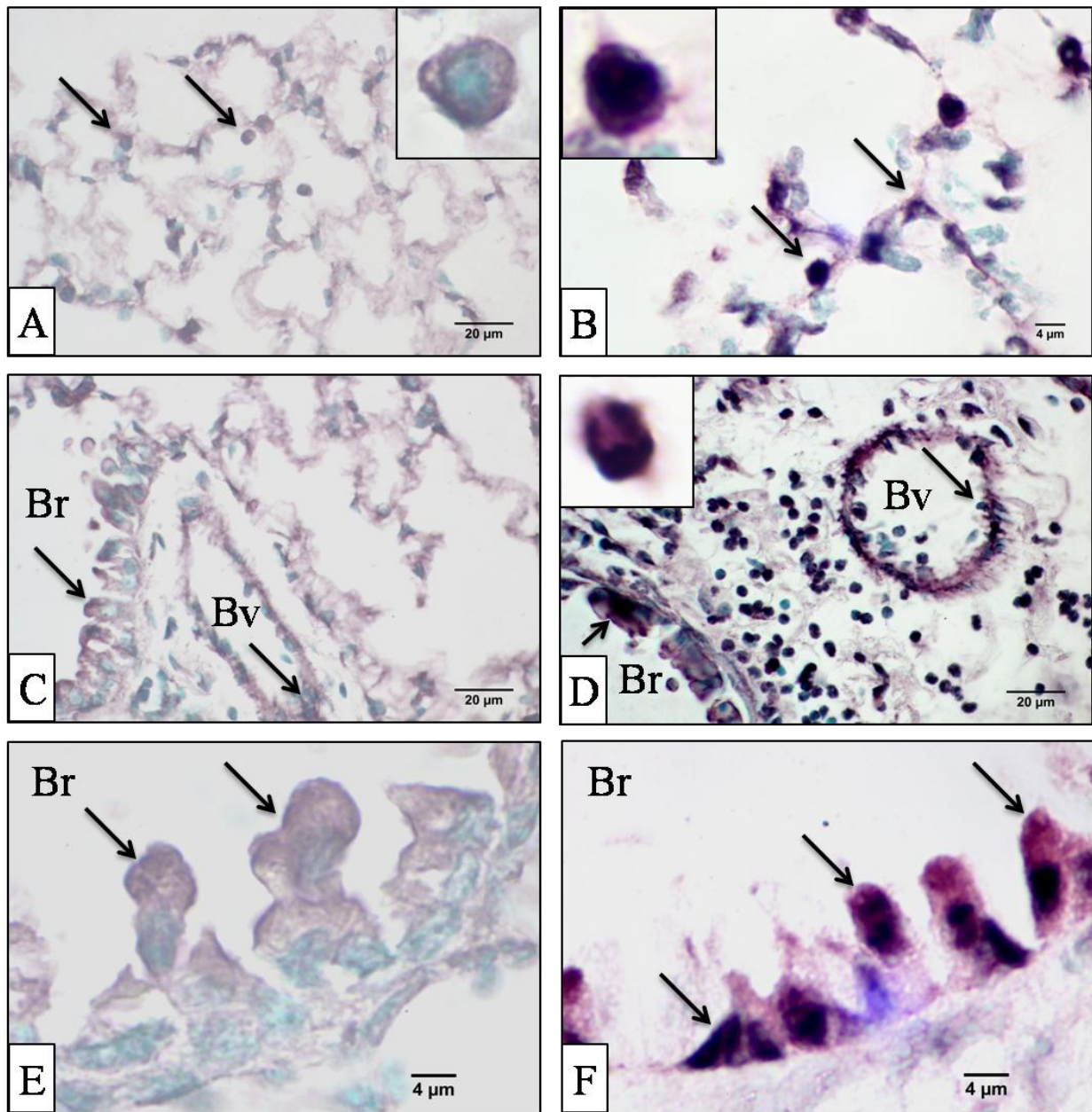


Figure 7.5. Mouse lung immunohistochemistry

Saline-treated WT mouse lung shows basal expression of LSP1 (arrows) in alveolar septum (A), macrophage (A, inset), bronchiolar epithelium (Br; C, E) and vascular endothelium (Bv, C). The LSP1 staining is much increased in at 9 hours post-LPS treatment in WT mouse lung in alveolar septum (B), macrophage (B, inset) and neutrophil (D, inset), vascular endothelium (D) and bronchiolar epithelium (Br; D, F). The high magnification views (E, F) show increased cytoplasmic and nuclear localization of LSP1 in bronchiolar epithelial cells. Abbreviation: Br, bronchiolar epithelium; Bv. Blood vessel. Original magnification 400 \times , 1000 \times . n=5 in each group

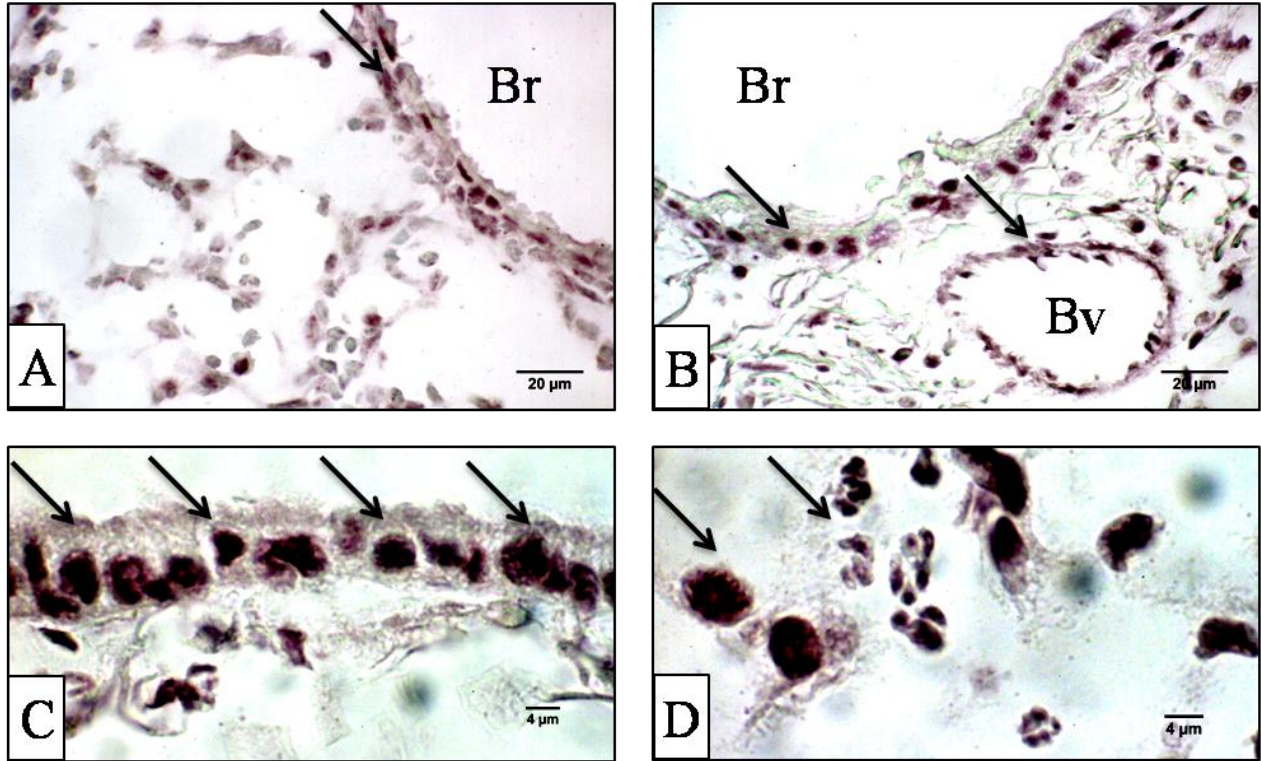


Figure 7.6. IHC for LSP1 in wild-type mouse lungs from 12-24 hour post- LPS treatment

LSP1 is expressed (arrows) in septum and bronchiolar epithelium (Br) and vascular (Bv) endothelium at 12 hours (A) and 24 hours (B) post-LPS treatment. Higher magnification views show nuclear staining of LSP1 in bronchiolar epithelium (C), macrophages and neutrophils (D) at 24 hour post-LPS treatment. Abbreviation: Br, bronchiolar epithelium; Bv. Blood vessel. Original magnification 400 \times , 1000 \times . n=5 in each group

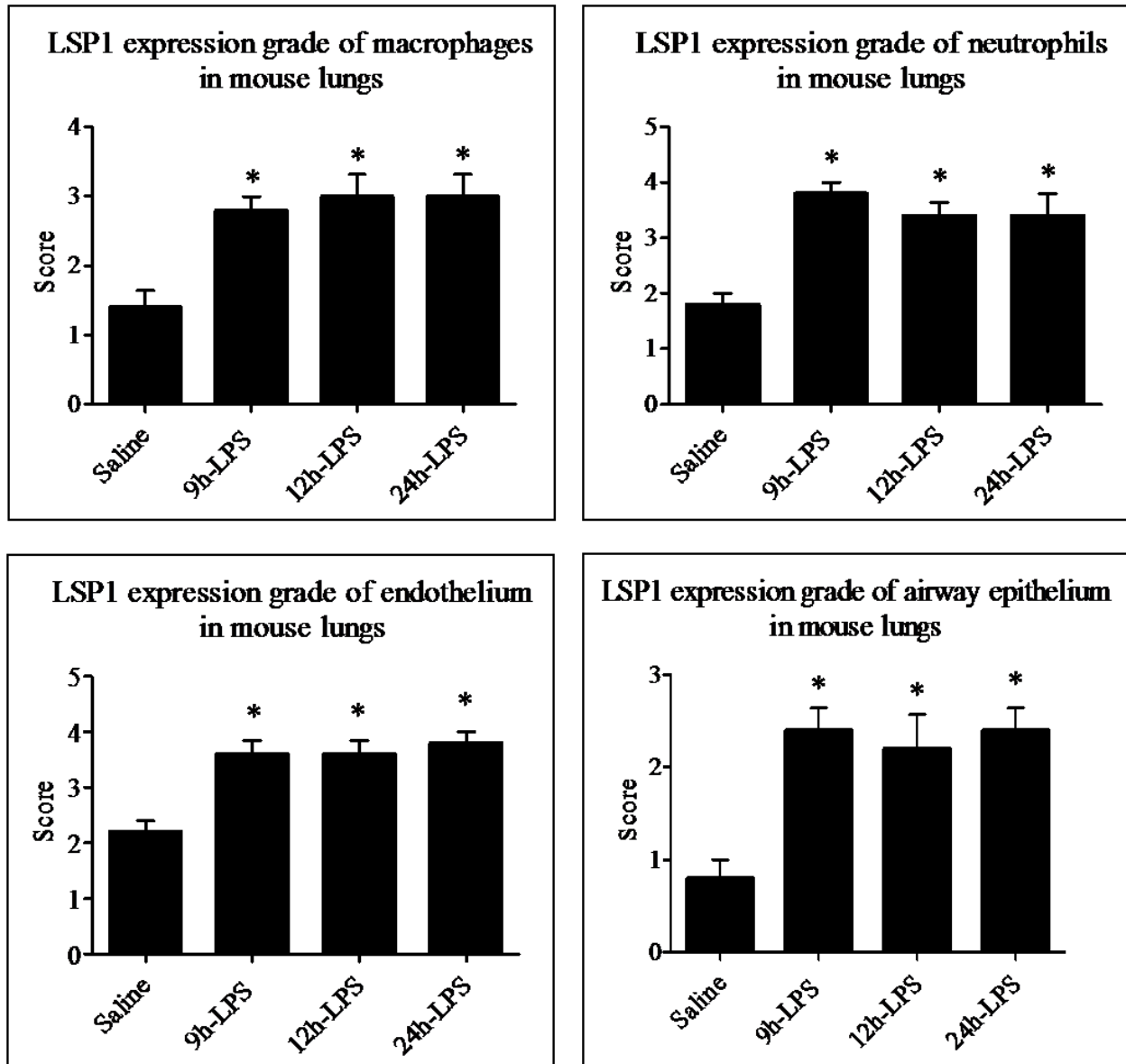


Figure 7.7. Statistical analysis semi-quantitative LSP1 expression scoring of wild-type mouse lung sections

In general, LSP1 expression increases in macrophage, neutrophils, endothelium and airway epithelium after LPS challenging 9, 12, 24 hours comparing to saline control. We did not observe any neutrophils in alveolar space and peribronchial vascular space in saline normal group. We classified that: 0: no staining, 1+: light staining, 2+: moderate staining, 3+: strong staining, 4+: very strong staining. Asterisk (*) indicates significant difference from wild-type saline ($P < 0.05$). $n = 5$ each group.

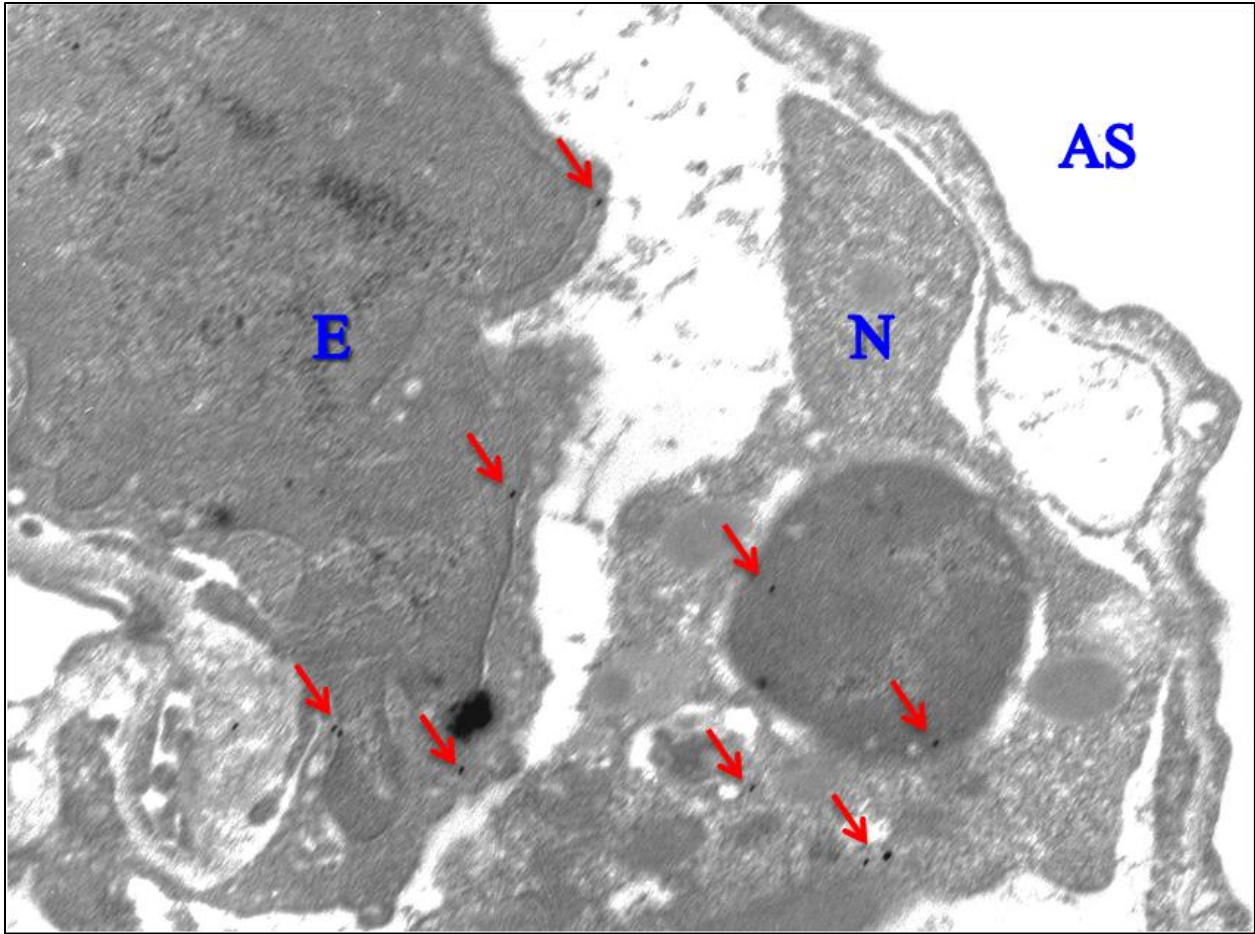


Figure 7.8. Immuno-gold electron microscopy for LSP1 in a mouse lung

The section from a mouse lung treated with LPS shows staining for LSP1 (arrows) in an endothelial cell (E) and neutrophil (N). AS: Alveolar space. Original magnification 13,000 \times .

Because of lack of frozen tissues from all the time points, we were able to compare only the LSP1 expression via western blots in normal and the 9 hour post-LPS mice. While western blots confirmed lack of LSP1 protein in the LSP1^{-/-} mice (Figure 7.9), the expression of phosphorylated as well as total LSP1 was significantly increased upon LPS treatment (Figure 7.9).

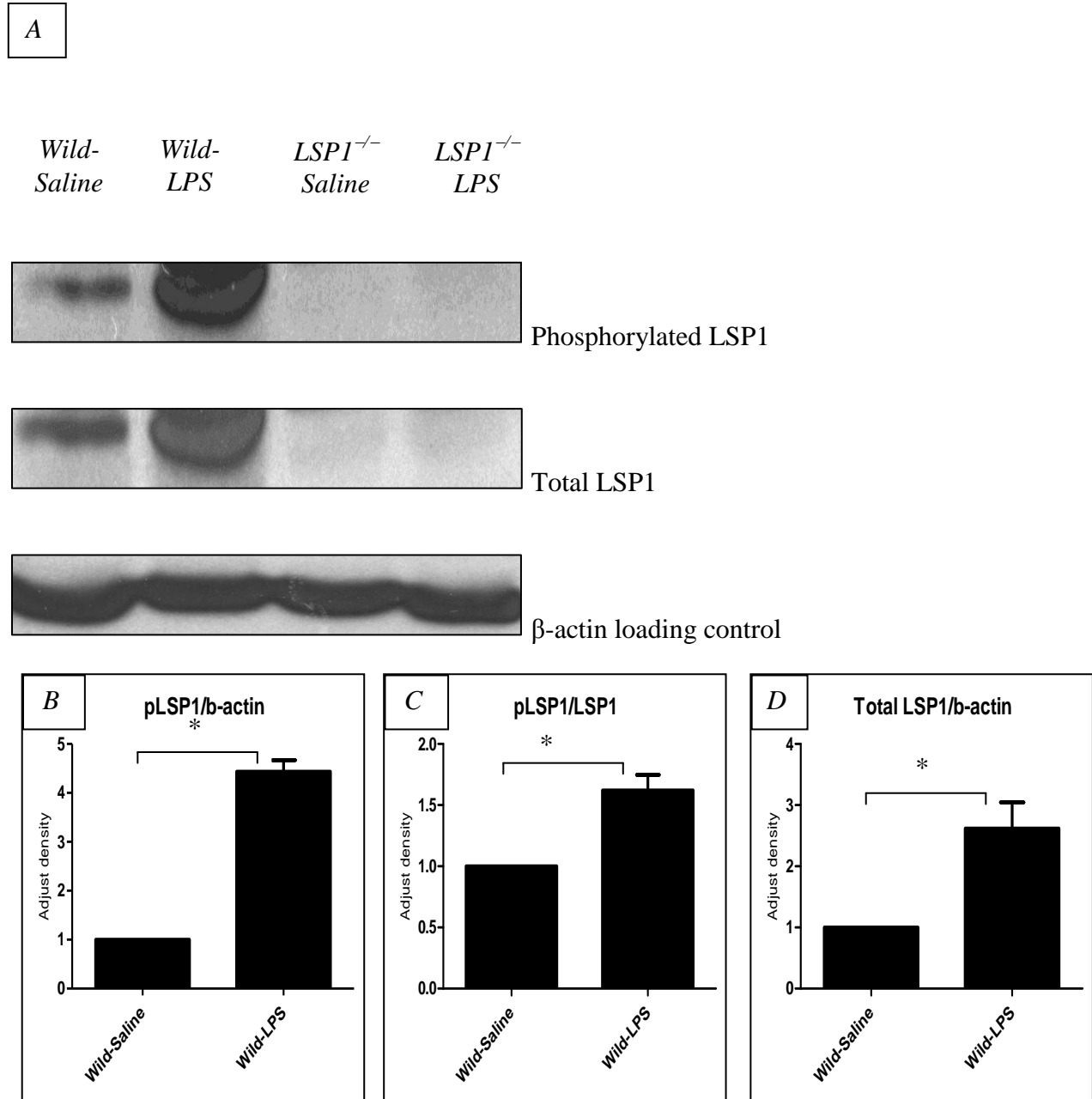


Figure 7.9. The *LSP1* observation in mouse lungs (A) after saline or LPS 9 hour treatment was confirmed by western blot following by densitometry quantification

Mouse lung pLSP1 and total LSP1 express one major band at about 52 kD. β -actin was used as a housekeeping gene which is not altered by any treatments. This protein serves as a loading control, identical amounts of whole cell lysates. β -actin observed band size is about 47 kD. Adjusted density of the relative abundance of pLSP1 over total LSP1 was shown in (C). Relative density of pLSP1 (B) and total LSP1 (D) were corrected for β -actin levels within each sample. Data were expressed as mean \pm standard error ($n=6$ each group). Asterisk (*) indicates significant difference from wild-type saline ($P<0.05$).

7.1.3. LSP-1 expression in human peripheral blood neutrophils

We further probed isolated human neutrophils for LSP1 expression with immuno-gold electron microscopy. Compared to the control neutrophils that showed LSP1 in their nucleus and cytoplasm (Figure 7.10), the LPS-treated neutrophils showed more staining for LSP1 in their nucleus and cytoplasm (Figure 7.11). In addition, there was increased LSP1 staining on the plasma membrane of LPS-treated neutrophils (Figure 7.12), which is similar to previous report in transformed B lymphoma cell line and normal thymocytes (96).

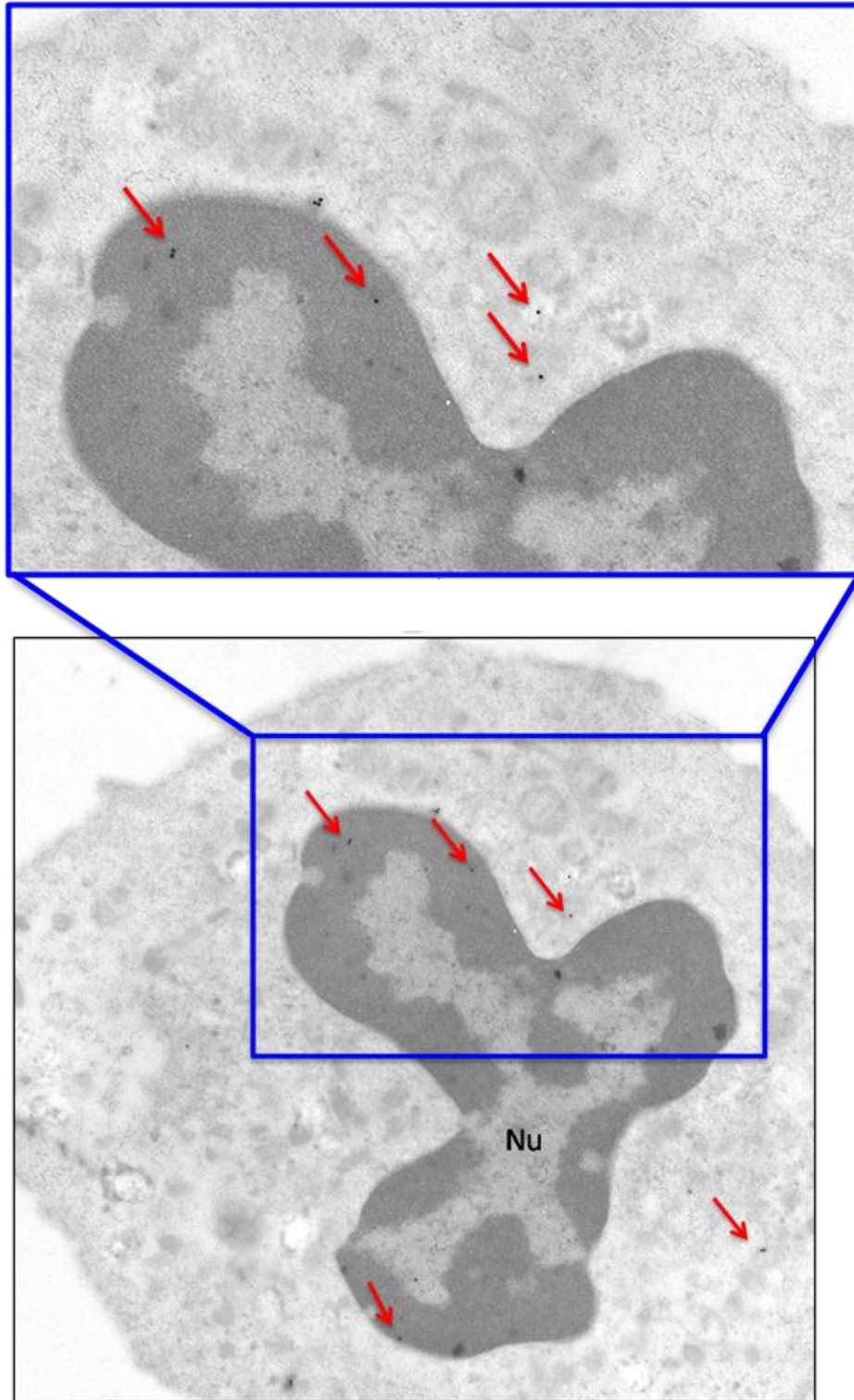


Figure 7.10. Immuno-gold electron microscopy for LSP1 for control neutrophil

Saline-treated neutrophil shows LSP1 staining (arrows) in nucleus (Nu) and cytoplasm. The boxed area magnified above shows LSP1 staining. Original magnification 13,000 \times .

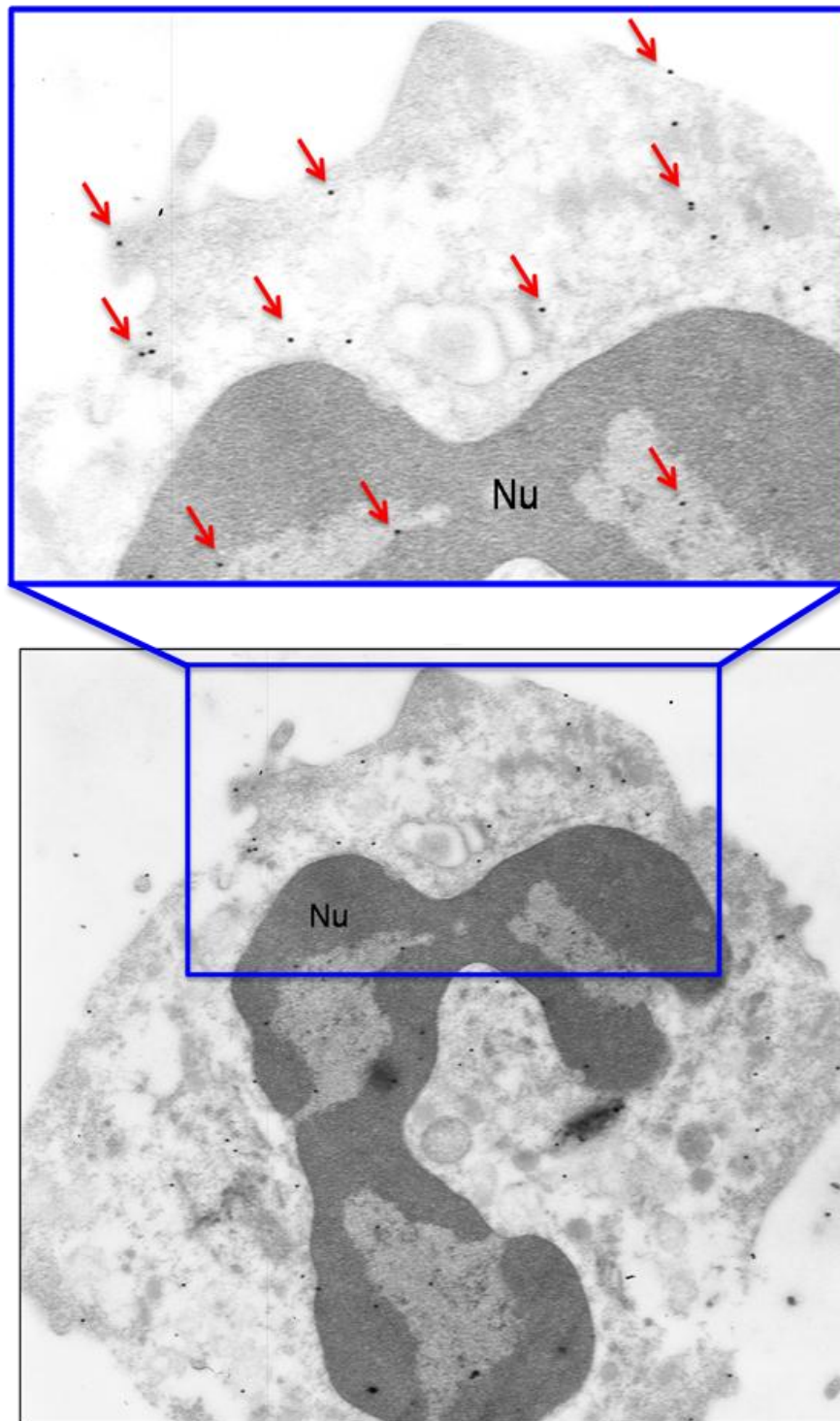


Figure 7.11. Immunogold electron microscopy for LSP1 for LPS-treated neutrophil

LPS-treated neutrophil shows increased LSP1 staining (arrows) compared to the saline-treated neutrophil (Figure 7.10) in nucleus (Nu) and cytoplasm. The boxed area magnified above shows LSP1 staining. Original magnification 13,000 \times .

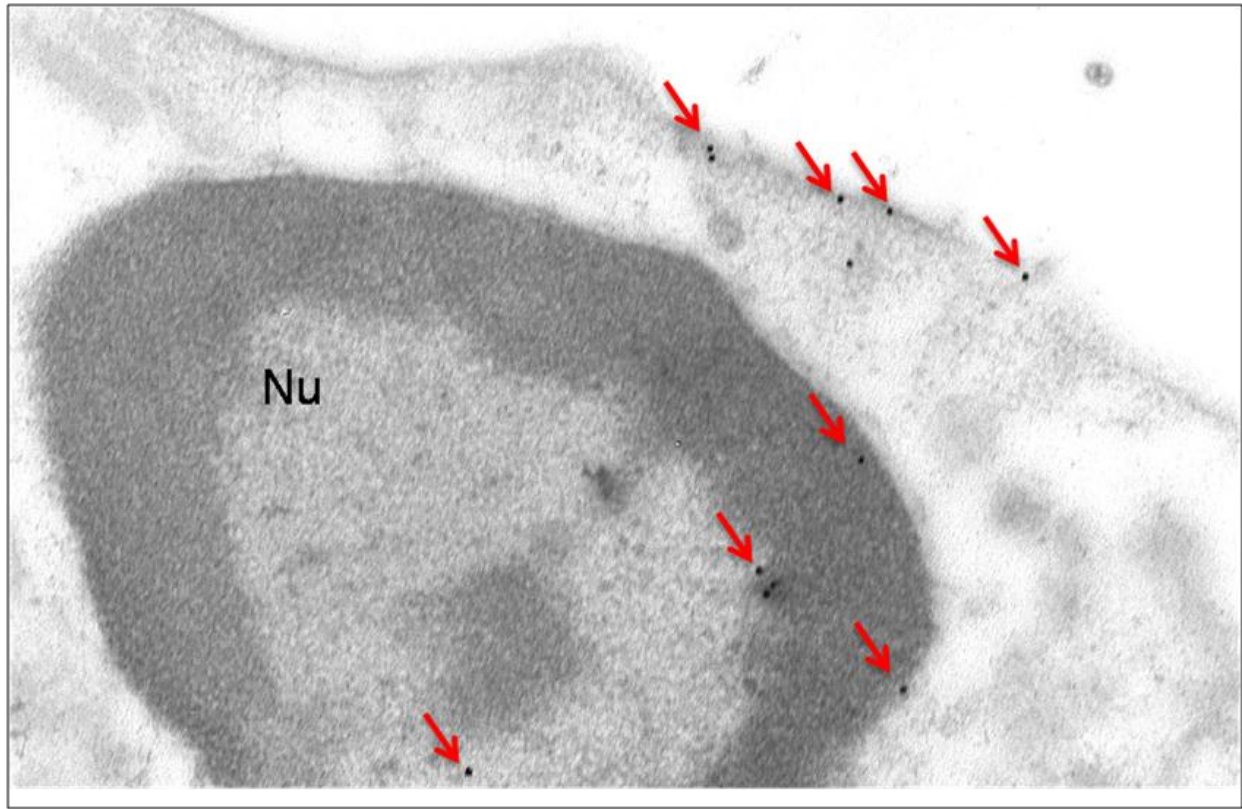


Figure 7.12. Immunogold electron microscopy for LSP1 for LPS-treated neutrophil

LPS-treated neutrophil shows increased LSP1 staining (arrows) on plasma membrane and the nucleus (Nu). Original magnification 13,000×

7.2. Examine the role of LSP1 in LPS-induced acute lung inflammation in mouse model

7.2.1. LSP1 deficiency reduces histologic signs of lung inflammation

First, we conducted histopathology assessment on lungs harvested from the mice in this study. As shown in hematoxylin and eosin staining pictures (Figure 7.13, Figure 7.14), while the lungs from saline-treated WT or LSP1^{-/-} mice showed normal architecture, the LPS-treatment induced congestion, damage to epithelium and infiltration of neutrophils in the septa and perivascular spaces. The lungs from LSP1^{-/-} mice treated with LPS showed considerable less inflammation and histopathology compared to LPS-treated WT mice. The extent of differences was also indicated by the fact that even though the lungs were lavaged, the lungs from WT-LPS mice compared to LSP1^{-/-}-LPS mice had many neutrophils in the alveoli.

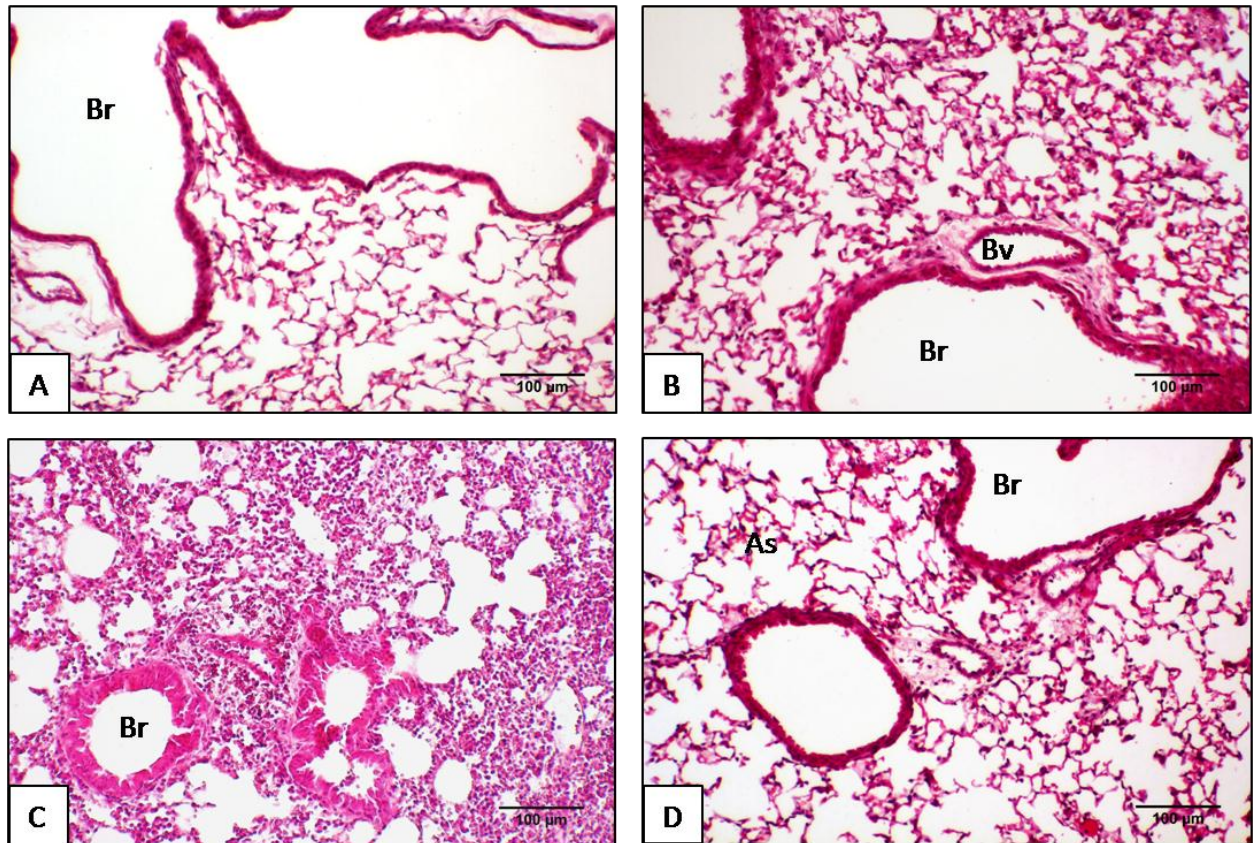


Figure 7.13. Histopathological examination of mouse lungs stained with hematoxylin and eosin

Lungs from wild-type (A) and $LSP1^{-/-}$ mice (B) treated with saline shows no inflammation and normal architecture. Wild-type mouse lung 9 hour post LPS (C) shows more severe inflammatory than $LSP1^{-/-}$ mouse lung challenged LPS (D) as indicated by thickening of alveolar septum due to more neutrophil accumulation. (As: Alveolar space, Bv: Blood vessel, Br: Bronchiole, PVS: Peribronchial vascular space). Original magnification 100×

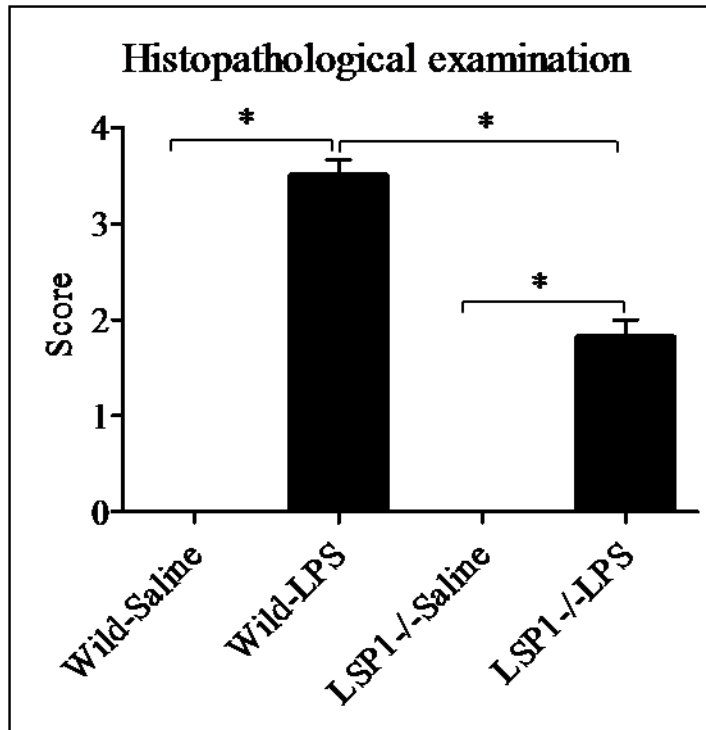


Figure 7.14. Statistical analysis semi-quantitative histopathology scoring of inflammation reaction in mouse lungs after saline or 9-hour LPS treatment

Asterisk (*) indicates significant difference ($P < 0.001$). Lesions were scored for both severity of inflammatory cell infiltrate and distribution: 0 (normal), 1 (minimal), 2 (mild), 3 (moderate), 4 (severe) as described above.

7.2.2. LSP1 deficiency reduces neutrophils recruitment into inflamed lungs

We examined the role of LSP1 in LPS-induced migration of neutrophils into lung alveoli. As shown in Figure 7.15, while there were no differences in total and different leukocytes count in peripheral blood between saline-treated WT and LSP1^{-/-} mice, peripheral blood neutrophil percentage were higher in WT and LSP1^{-/-} mice at 9 hour post-LPS treatment compared to their respective controls ($P < 0.001$).

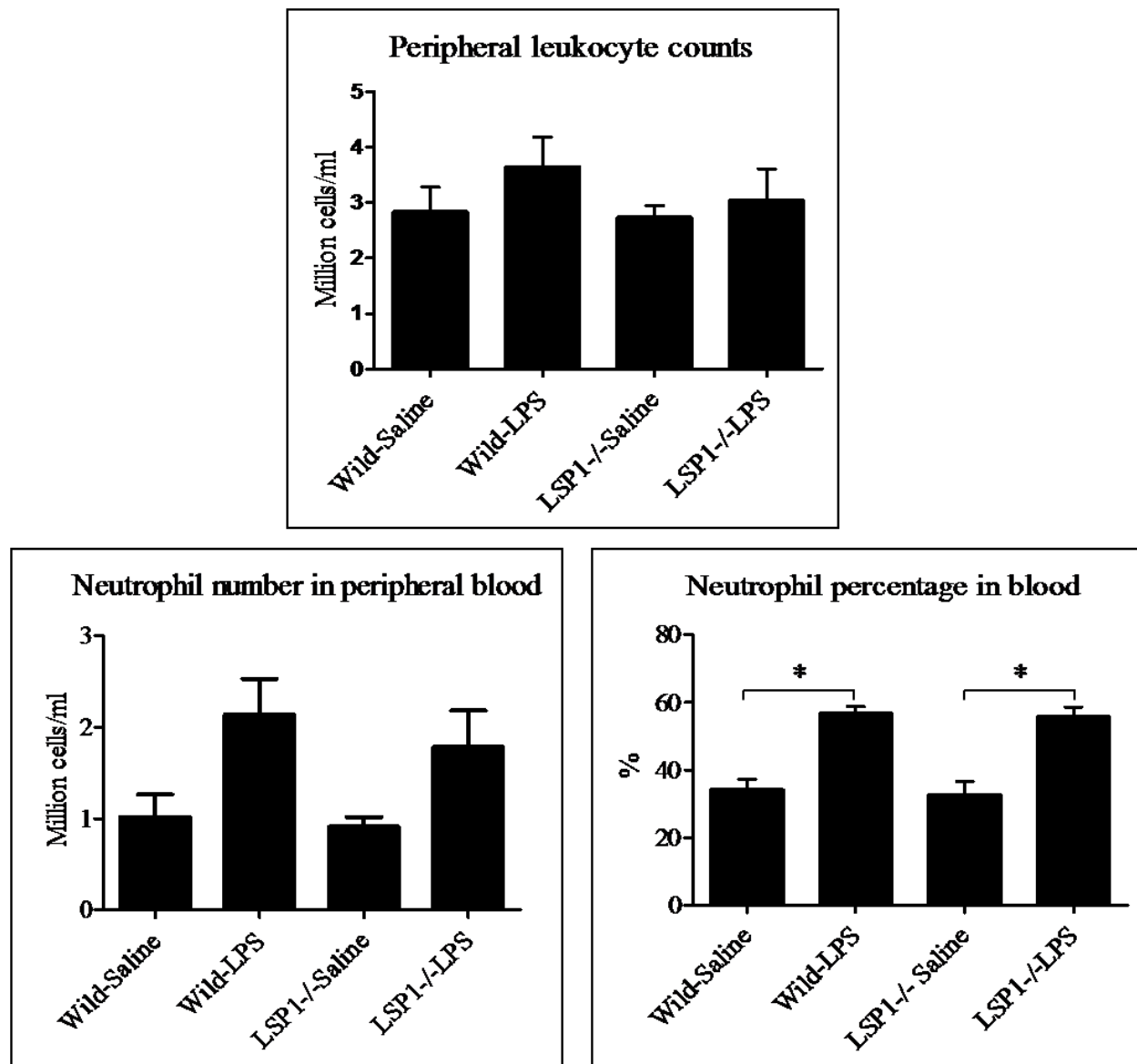


Figure 7.15. Peripheral leukocyte count data

LSP1^{-/-} does not affect peripheral blood cell quantity in LPS-treated mice. However, we observed that the number of neutrophils in peripheral blood of wild-type mice was little higher than that of LSP1^{-/-} mice after 9 hour LPS treatment. Neutrophilia was observed in both phenotypes of mice. Asterisk () indicates significant difference ($P < 0.001$).*

As depicted in Figure 7.16, LPS treatment increased the numbers of total cells in BAL fluid of WT and LSP1^{-/-} mice compared to their respective controls (WT groups $P < 0.001$, LSP1^{-/-} groups $P < 0.05$). Furthermore, the total cell counts were higher in BAL fluid from WT-LPS mice compared to LSP1^{-/-}-LPS mice ($P < 0.001$). Similarly, the absolute neutrophil numbers but not monocyte numbers were significantly higher in BAL fluid of WT-LPS mice compared to LSP1^{-/-}-LPS mice ($P < 0.001$).

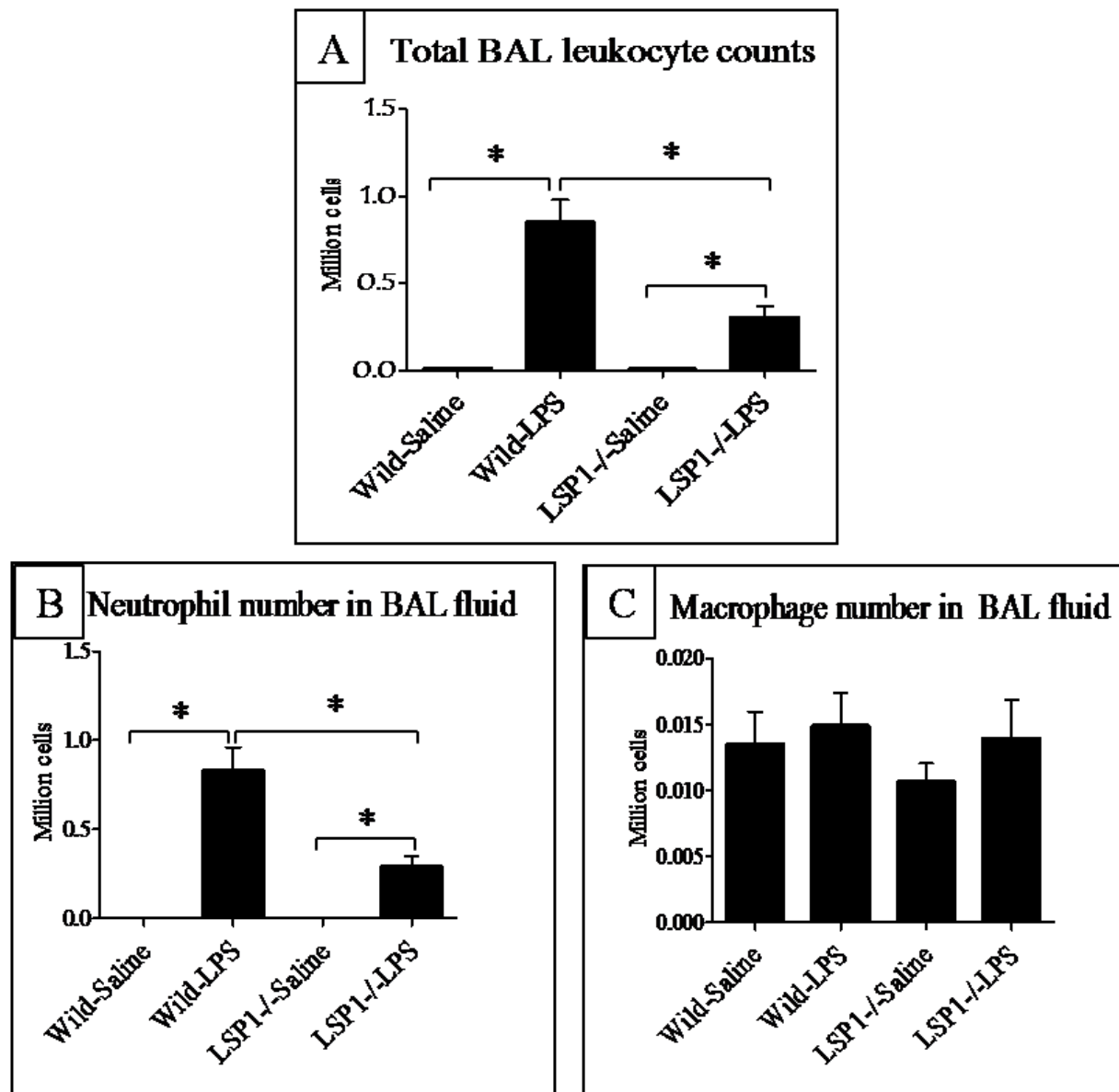


Figure 7.16. Total and differential leukocyte count in bronchoalveolar lavage fluid

Compared with saline treatment, a substantial increase in total leukocyte (A), mainly neutrophil (B) in the airway after 9 hours post-LPS intranasal instillation was observed in both phenotypes of mice. LSP1 deficiency reduces recruitment of neutrophils in LPS-treated lungs in comparison with wild-type. There was an increase but not significant change in the number of macrophages in bronchoalveolar lavage fluid (C). Asterisk (*) indicates significant difference.

It is known that many neutrophils are trapped in the vasculature or interstitium of inflamed lungs in addition those that have migrated into the alveoli and are flushed with BAL. Therefore, we used MPO estimation as a surrogate for the lung tissue neutrophils. As described in Figure 7.17, compared to respective saline controls, higher MPO activity was detected in lungs of WT-LPS ($P < 0.001$) but not $LSP1^{-/-}$ -LPS mice. Lungs from WT-LPS mice also had higher MPO activity compared to $LSP1^{-/-}$ -LPS mice ($P < 0.05$). We obtained morphological evidence of presence of neutrophils in lung tissues with Gr-1 immunohistochemistry (Figure 7.18). The data showed more neutrophils in lung sections from WT-LPS mice than from $LSP1^{-/-}$ -LPS mice (Figure 7.19).

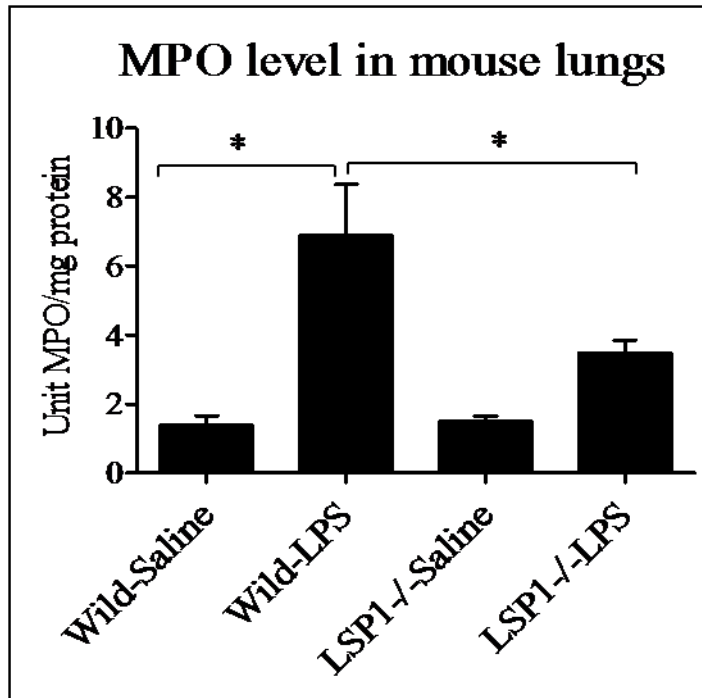


Figure 7.17. Myeloperoxidase content in broncho-alveolar lavage fluid

LSP1 induced myeloperoxidase activity in LPS administrated lungs. Asterisk (*) indicates significant difference.

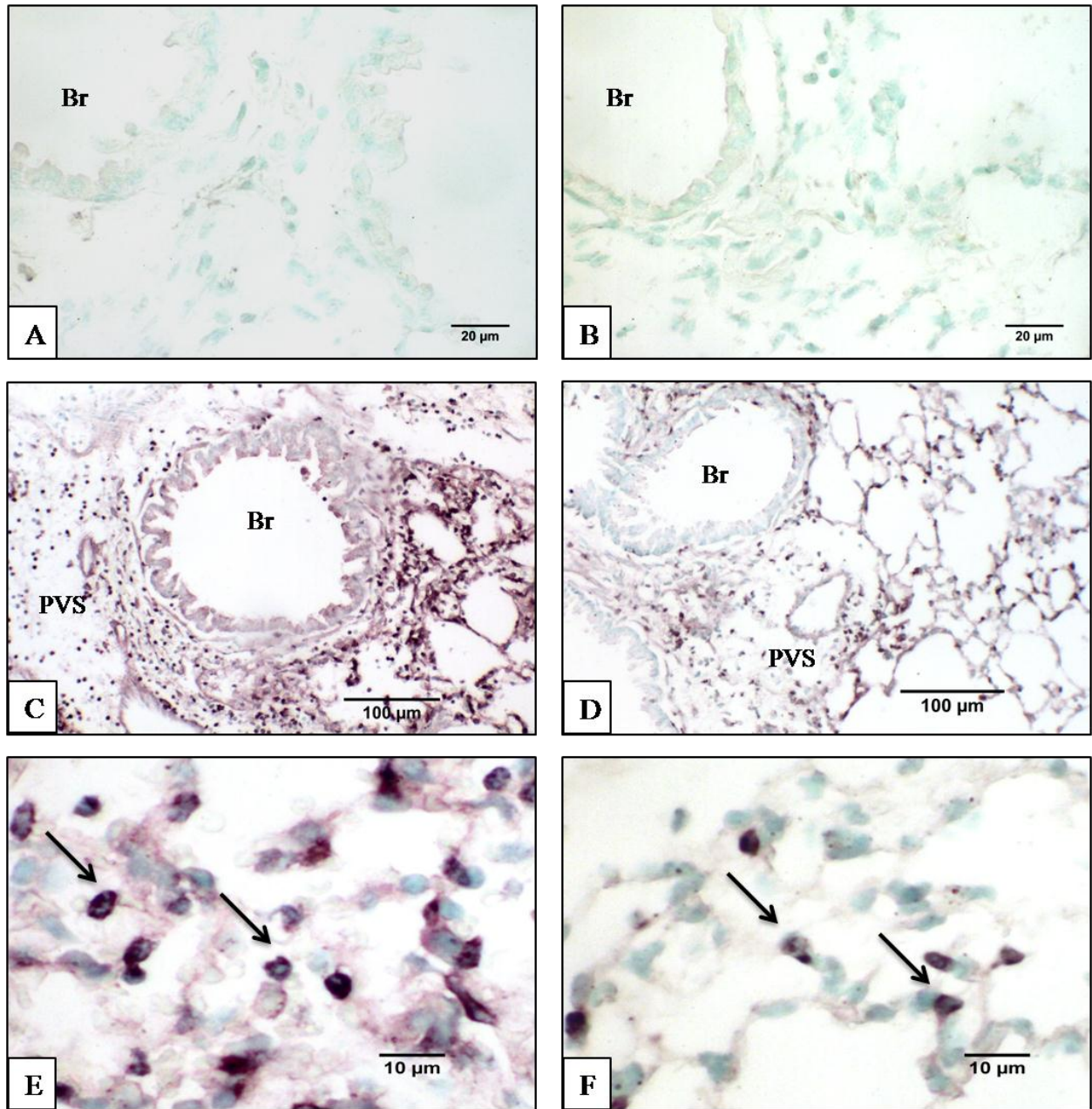


Figure 7.18. Representative pictures of Gr-1 immunohistochemistry staining of mouse lungs

Lungs from wild-type (A) and $LSP1^{-/-}$ mice (B) treated with saline show only occasional neutrophils. Wild-type mouse lung 9 hour post LPS administration (C, E) shows more neutrophils were trapped in the airway than $LSP1^{-/-}$ mouse lung (D, F) after 9 hour LPS instillation. $LSP1^{-/-}$ reduces neutrophil accumulation. (As: Alveolar space, Bv: Blood vessel, Br: Bronchiole, PVS: Peribronchial vascular space). Original magnification 400 \times , 1000 \times . n=5 each group.

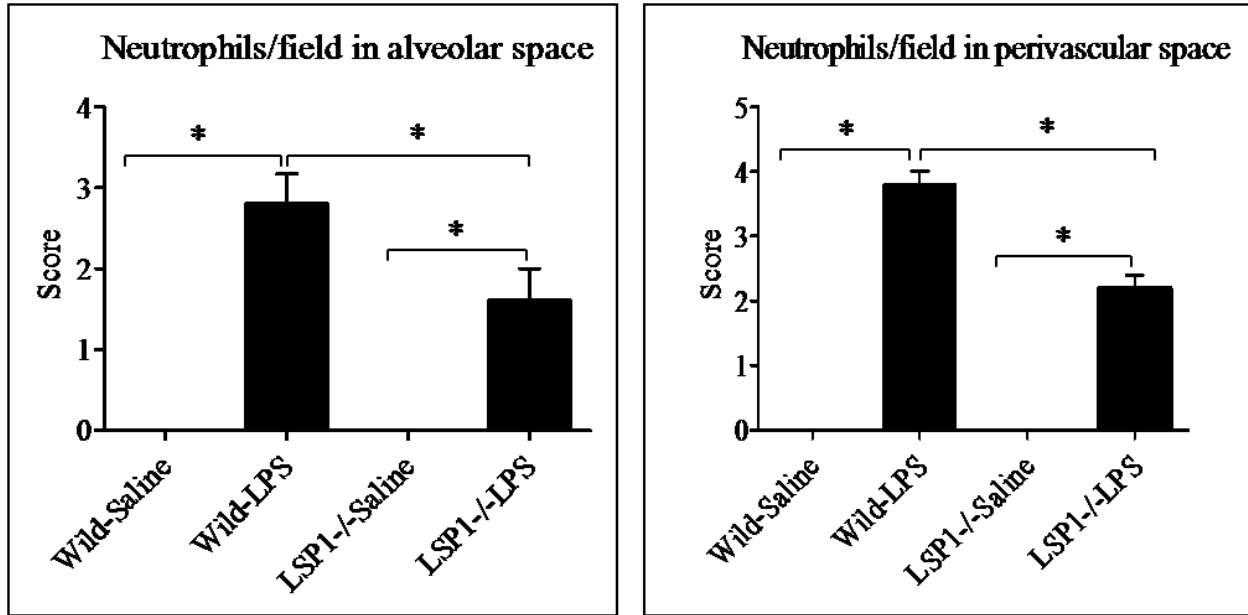


Figure 7.19. Semiquantitative representation of Gr-1 lung immunohistochemical staining

Acceleration of neutrophil infiltration in the lungs at 9-hour after LPS treatment was evidenced by grading Gr-1-positive cells per 1000X field. Data shows preventive effects of LSP1 deficiency towards neutrophils infiltration in lungs undergone 9 hour LPS treatment.

7.2.3. LSP1 deficiency reduces expression of TNF- α but not KC, MCP-1, MIP-1 α , MIP-1 β in BAL

To determine the mechanisms underlying the effects of LSP1 on neutrophil migration into inflamed lungs, we quantified KC, MCP-1, MIP-1 α , MIP-1 β , and TNF- α in BAL fluid and lung homogenates. As demonstrated in Figure 7.20, while there were no differences in KC concentrations in BAL fluid of LPS-treated mice compared to their respective controls, TNF- α , MIP-1 α , MIP-1 β were significantly increased following LPS-treatment of both WT and LSP1^{-/-} mice compared to their respective controls (P<0.05). MCP-1 in BAL fluid was increased in WT but not LSP1^{-/-} mice following LPS treatment. Except TNF- α (P<0.05), there were no differences in the concentrations of any of the other inflammatory mediators between the WT and LSP1^{-/-} mice treated with LPS.

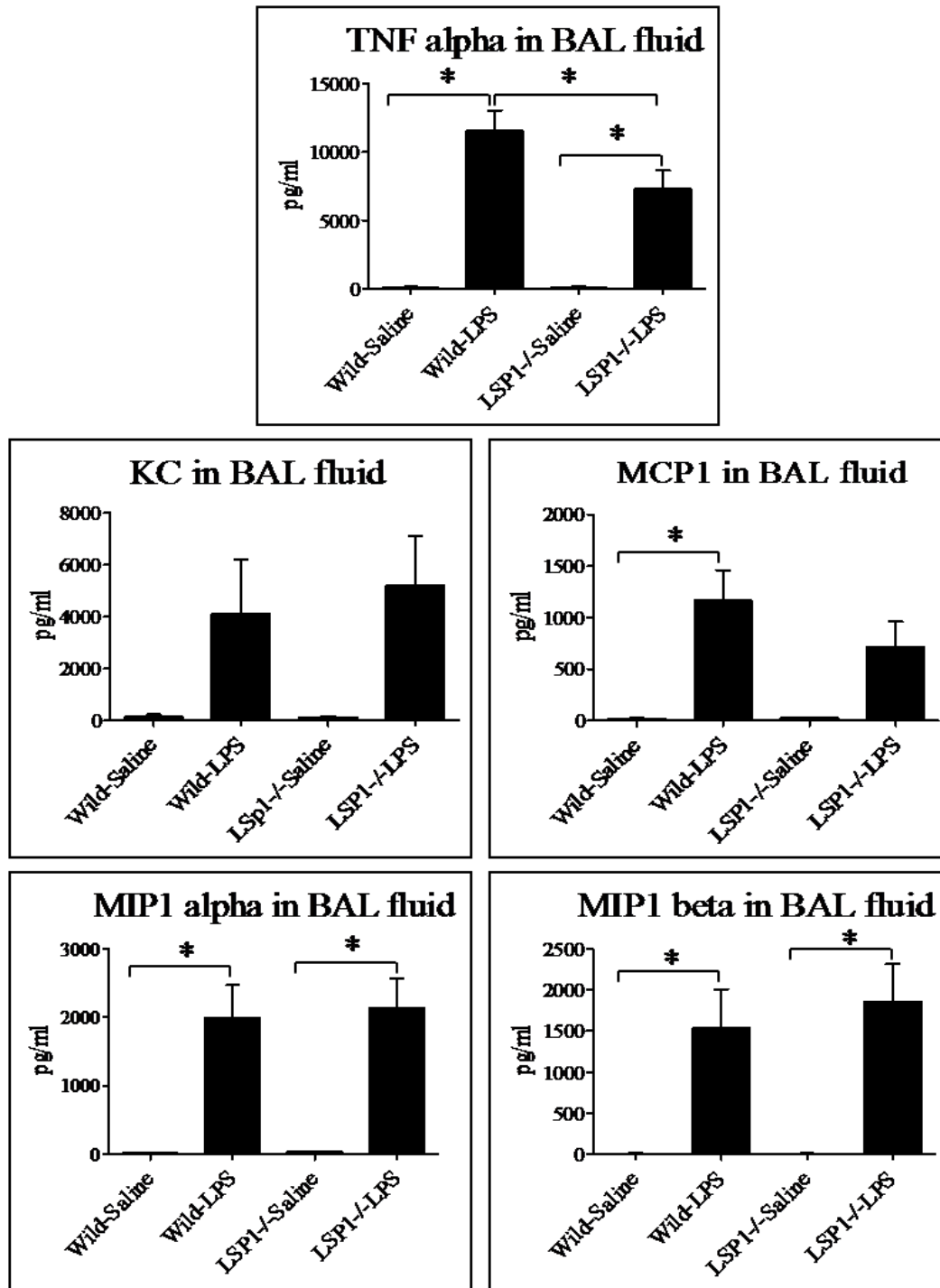


Figure 7.20. Proinflammatory cytokine and chemokine productions in BAL fluid

LSP1 enhance TNF- α in BAL fluid. Asterisk () indicates significant difference. Abbreviation: TNF- α : Tumor necrosis factor- α , KC: Keratinocyte-derived chemokine, MCP-1: Monocyte chemoattractant protein 1, MIP-1: macrophage inflammatory protein 1.*

7.2.4. LSP1 deficiency did not affect expression of TNF- α , KC, MCP-1, MIP-1 α , MIP-1 β in lung homogenates

In lung tissues, the LPS treatment of WT and LSP1^{-/-} mice significantly increased concentrations of KC, MCP-1, MIP-1 α , MIP-1 β , and TNF- α compared to their respective saline controls ($P < 0.05$). There, however, were no differences in the concentrations of these inflammatory mediators between WT and LSP1^{-/-} mice in the normal or LPS-treatment (Figure 7.21).

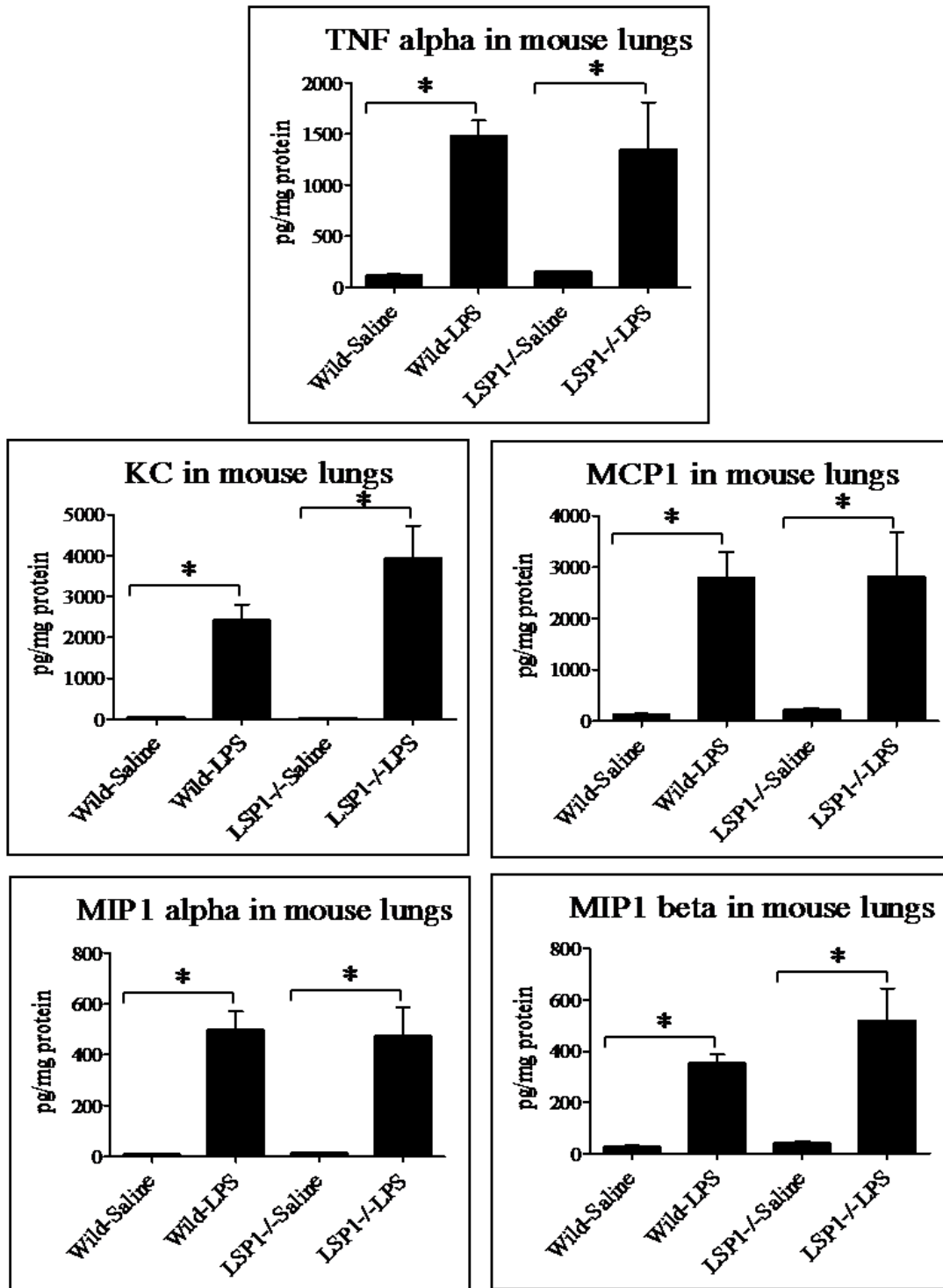


Figure 7.21. Proinflammatory cytokines and chemokines in mouse lung homogenates

Lungs of both mouse phenotypes treated with LPS show significant higher TNF- α , KC, MCP-1, MIP-1 α , and MIP-1 β in comparison with saline negative control, respectively. LSP1 did not affect chemokine in remaining lung tissue. Asterisk (*) indicates significant difference.

7.2.5. LSP1 deficiency does not regulate vascular permeability in inflamed lungs

In the LPS administered animals, protein concentration in BAL fluid increased significantly compared to saline control in both types of mice ($P < 0.01$) (Figure 7.22). However, there was no significant difference in protein level between wild-type and LSP1^{-/-} mice after treatment with LPS.

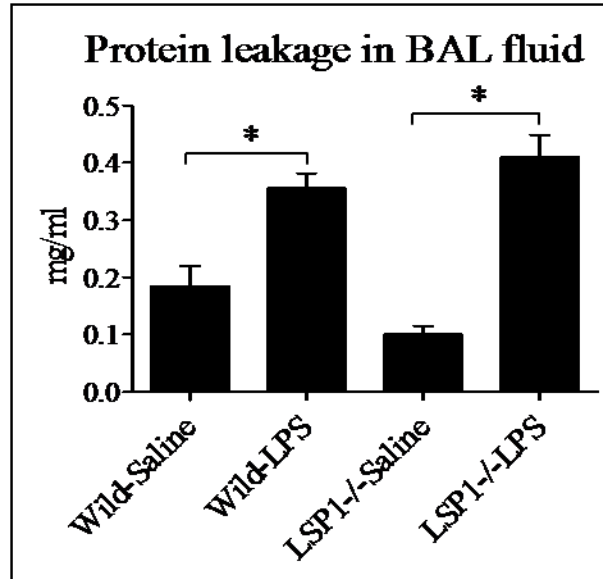


Figure 7.22. Protein concentration in bronchoalveolar lavage fluid

Both types of mice challenged with LPS showed significantly higher protein content than saline control. LSP1 did not affect substantially total protein leakage in BAL fluid at this time point. Asterisk (*) indicates significant difference.

7.2.6. LSP1 deficiency affects phosphorylation of p38MAPK in inflamed lungs

MAPKAP2 as well as p38MAPK was known as an upstream substrate for LSP1 (11, 90). Inhibition of p38MAPK can reduce endothelial LSP1 phosphorylation (91). Therefore, in our study, densitometric quantification of Western blots for phosphorylated Thr180/Tyr182 p38MAPK and total p38MAPK of mouse lung in saline and LPS 9 hour administration was performed (Figure 7.23). Densitometry quantification data (Figure 7.23) shows that in wild-type groups but not LSP1^{-/-} groups, phosphorylated p38MAPK level increased at 9 hour LPS treatment compared with saline control (P<0.05). There was no significant difference between wild-type and LSP1^{-/-} mice regarding the adjust density of phosphorylated p38MAPK and p38MAPK.

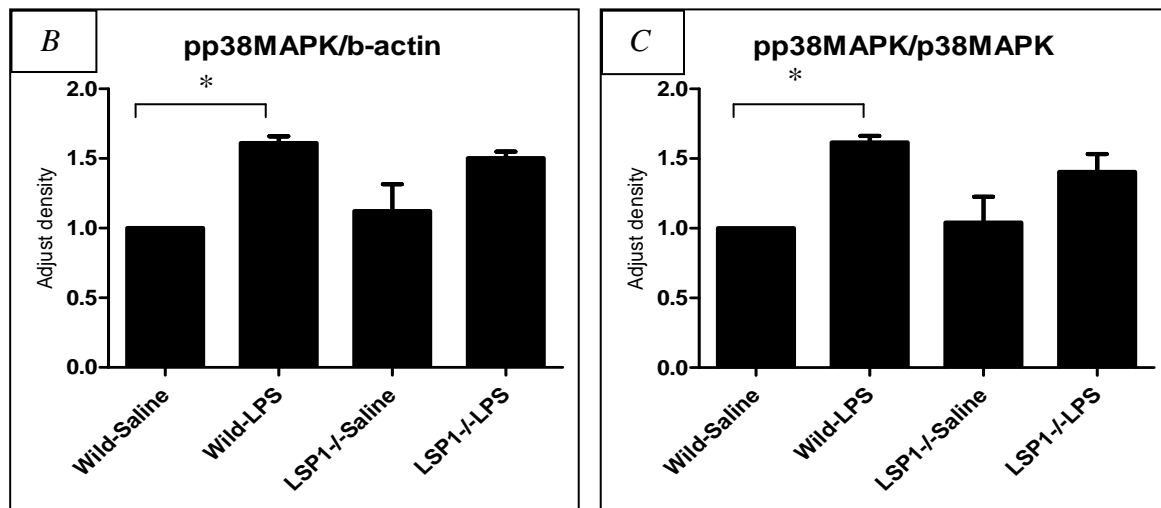
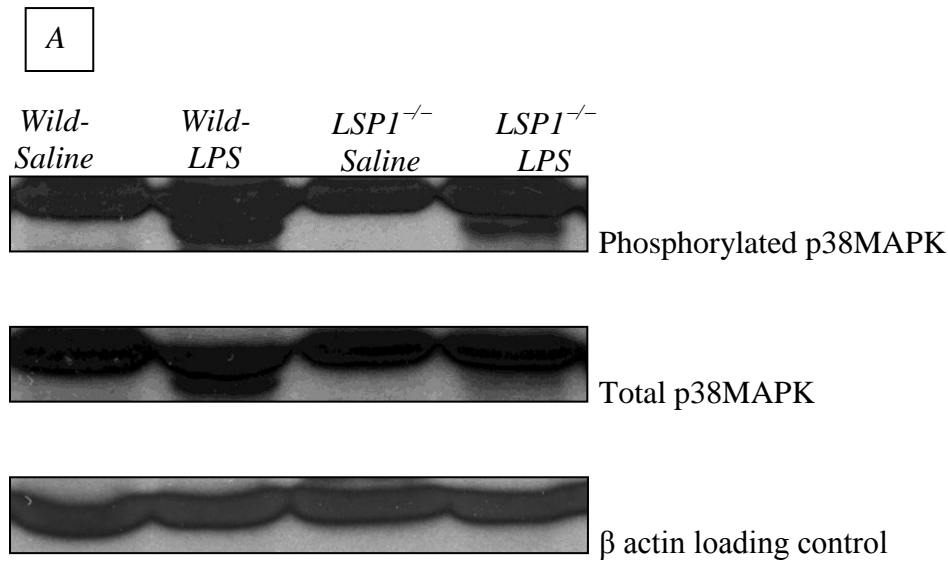


Figure 7.23. Western blots (A) for phosphorylated p38MAPK and total p38MAPK of mouse lungs after saline or LPS 9 hour administration following by densitometry quantification.

pp38MAPK and p38MAPK molecular weight are about 43 kD and 38 kD, respectively. β -actin was used as a housekeeping gene which is not altered by any treatments. This protein serves as a loading control, identical amounts of whole cell lysates. β -actin observed band size is about 47 kD. Relative density of phosphorylated p38MAPK was corrected for β -actin levels within each sample (B). Densitometry quantification of phosphorylated pp38MAPK over total p38MAPK in mouse lungs was depicted in (C). p38MAPK phosphorylation increased due to LPS treatment in both phenotypes of mice. Data were expressed as mean \pm standard error ($n=4$ each group). Asterisk (*) indicates significant difference from wild-type saline ($P<0.05$).

8. DISCUSSION

We provide evidence for a role for LSP1 in LPS-induced neutrophil migration but not vascular permeability in acute lung inflammation. The data show that LSP1 may be influencing lung inflammation through phosphorylation of p38MAPK and TNF- α expression but without altering expression of KC, MCP-1, MIP-1 α , and MIP-1 β . The expression of LSP1 was increased in lungs from LPS-treated mice and also in lungs from septic humans. The expression was especially intense in neutrophils and their nuclei. These data add to our understanding of mechanisms of neutrophil recruitment in inflamed lungs as well as biology of LSP1.

We used a described model of LPS-induced acute lung inflammation (116, 117). Studies on distribution of LSP1 protein on inflamed lung have been limited (1, 88, 100). We used immunohistology to show LSP1 in airway epithelium, alveolar macrophages and septal cells. The expression of LSP1 was increased in epithelium, endothelium and inflammatory cells including neutrophils. The increased expression of LSP1 in airway epithelium and inflammatory cells such as neutrophils was sustained up to 24 hours post-LPS treatment. The expression of LSP1 was also increased in autopsied lungs from septic humans. The immunohistologic increase was confirmed with Western blots on mouse lung tissue homogenates. The expression of total LSP1 protein was significantly increased at 9 hours post-LPS treatment. Within the total LSP1, phosphorylated LSP1 was also significantly increased in lung homogenates from LPS-treated mice to suggest activation of LSP1. Taken together, our data showed that lung inflammation alters expression of LSP1 and increases the amount of phosphorylated LSP1.

Immunohistologic data showed intense expression of LSP1 in neutrophil nuclei in autopsied septic lungs. Interestingly, there is very limited information available on the subcellular localization of LSP1 (101, 124). Therefore, recognizing the limiting resolution of light micro-

scope, we used immuno-gold electron microscopy to show that LSP1 is present on the plasma membrane and nucleus of lung vascular endothelium and neutrophils. Some authors proposed that LSP1 interacted with cytoskeleton through F-actin binding, which have functions in cell motility and inter-cellular interactions (124). It is also believed that endothelial LSP1 can translocate from nucleus and cytosol to the cytoskeleton to form endothelial domes to allow neutrophils transendothelial migration (101). Although our studies do not inform on the role of nuclear LSP1, the data showing increased staining of LSP1 in neutrophils in inflamed lungs creates a need to further clarify the mechanisms and the reasons for the movement of LSP1 into the nucleus.

Endotoxin-induced lung injury is characterized by migration of neutrophils into lung tissues, and migration of activated neutrophils, though necessary for host defense, is credited with excessive tissue damage. Therefore, significant scientific effort is underway to more precisely understand the molecules regulating neutrophil migration into inflamed lungs. There are data to show the role of selectins and integrins (67, 69), along with various chemokines, in multi-step paradigm of recruitment of neutrophils into the lungs (45, 74, 125-127). Our data show that LSP1^{-/-} reduces lung pathology and also influences migration of neutrophils into inflamed lungs. LSP1 deficiency reduced migration of neutrophils into BAL and also into lung vascular and interstitial compartments based on analyses of BAL and lung tissues. Lung tissue neutrophil content was also assessed through myeloperoxidase quantification and staining of lung tissues with Gr-1 antibody as previous papers (128-131). Taken together, these data suggest that LSP1 influences tissue and alveolar recruitment of neutrophils into inflamed lungs. The reduced alveolar and tissue migration of neutrophils suggests an interference of LSP1 at the level of interaction of endothelium and neutrophils as both of these cells express LSP1. Such a contention is supported

by whole-mount staining of cremaster muscle tissue or 2-photon microscopy showed a double-fold increase in the quantity of domes in wild-type mice than that in LSP1^{-/-} mice after TNF- α or IL-1 β administration (101). Moreover, transmission electron microscopy technique illustrated that endothelial domes completely enveloped neutrophils in wild-type but not in LSP1^{-/-} mice (101). We demonstrate localization of LSP1 on the plasma membranes of neutrophils and endothelial cells, and speculate whether LSP1 is involved in direct physical engagement of endothelium with the neutrophils.

We aimed to explore the mechanisms through which LSP1 may be regulating migration of neutrophils. First, we examined phosphorylation of p38MAPK in lung homogenates because LPS instillation causes increased phosphorylated p38MAPK and inhibition of p38MAPK phosphorylation reduces lung inflammation and cytokine expression (132-136). Our data show reduced phosphorylated p38MAPK in LPS-treated LSP1^{-/-} mice compared the treatment matched WT mice. Interestingly, inhibition of phosphorylated p38MAPK leads to diminished phosphorylation of LSP1 (91). Therefore, endothelial LSP1 phosphorylation is clearly a downstream molecule of p38MAPK signaling, and the relationship between the two may underlie reduced migration of neutrophils in LSP1^{-/-} mice (23, 91, 101). These data need to be strengthened through studies of p38MAPK on normal and activated neutrophils isolated from wild-type and LSP1^{-/-} mice and the interaction of such neutrophils with isolated lung microvascular endothelium in future.

In this study, we demonstrated the LSP1 function in LPS-induced acute lung injury in mouse model by comparing normal mice and mice lack of LSP1. In saline control sham groups, mice lack of LSP1 developed normally and had the same results as wild-type mice. Therefore, the differences between wild-type and LSP1 deficient mice are the consequence of LSP1 absence

but not because of problem from deficient procedure of gene targeting. The immunoblot results indicated that wild-type and LSP1^{-/-} mice did not have a significant difference in phosphorylated p38MAPK. LSP1 phosphorylation augmented considerably after 9 hour LPS treatment. It is identified that the association of LSP1 with the cytoskeleton, especially F-actin in mouse bases on its basic COOH-terminal site (10) which is the phosphorylation area. The binding sites of over-express LSP1 with F-actin are in three main regions: amino acid 181-245, amino acid 246-295, and amino acid 306-339 (97). The site within the residues 231–330 binds LSP1 tightly with F-actin (6). In our study, we focus on LSP1 phosphorylation ser 252 in human, corresponding to ser 243 in mouse, which was proven a major phosphorylation site of LSP1 (11). Therefore, taken together, it can be concluded that LSP1 is one of the conditional requirements for neutrophil transmigration, an important stage involved acute lung inflammation mechanism.

To further understand the mechanisms of reduced neutrophil migration in LPS-treated LSP1^{-/-} mice, we examined expression of KC, MCP-1, MIP-1 α , MIP-1 β , and TNF- α , which have many important functions on acute lung injury (125-127). We found increased concentrations of KC, MCP-1, MIP-1 α , MIP-1 β , and TNF- α in lung homogenates of LPS-treated mice but there was no impact of LSP1 deficiency on their expression. The recruitment of neutrophils into alveoli is directed through chemokines and we examined KC, MCP-1, MIP-1 α , MIP-1 β , and TNF- α concentration in BAL fluid. Except TNF- α , there was no effect of LSP1 deficiency on secretion of these inflammatory mediators. In other words, this finding can suggest that TNF- α , a cytokine production of activated monocyte, macrophage (137), have more complex and important relationship with LSP1 in upregulating neutrophils infiltration than other cytokines and chemokines in this acute lung inflammation model. In other organs, neutrophil recruitment through post-capillary venules of LSP1^{-/-} mice is restricted even when activated by various cy-

tokines (TNF- α and IL-1 β) and chemokine (KC, MIP2) suggesting that endothelial LSP1 has a significant role in transendothelial migration of neutrophils (22, 23). It is interesting to note that while LPS-treated increased MCP-1 concentrations in the BAL of WT mice, there was no difference between the control and LPS-treated LSP1^{-/-} mice. The lack of upregulation of MCP-1 may have implications for the recruitment of monocytes/macrophages that occurs in the later parts of lung inflammation induced by a single challenge with LPS, and needs to be studied further.

Intriguingly, we found that LSP1 deficiency does not protect the lung against increased vascular permeability. While our study does not provide explanation for this observation, there are previous data to show that lung vascular permeability may be independent of neutrophil migration (81, 138). It has also been reported that every neutrophil immigrating in KC, TNF- α or MIP-2-treated cremaster muscles of LSP1^{-/-} mice caused significantly higher vascular permeability (22, 101, 115). Furthermore, while permeability reduction was known to be a result of leukocyte infiltration decrease in mesentery (139), it was reported that besides leukocyte emigration, there were other factors affect the increase of plasma leakage such as histamine (140, 141).

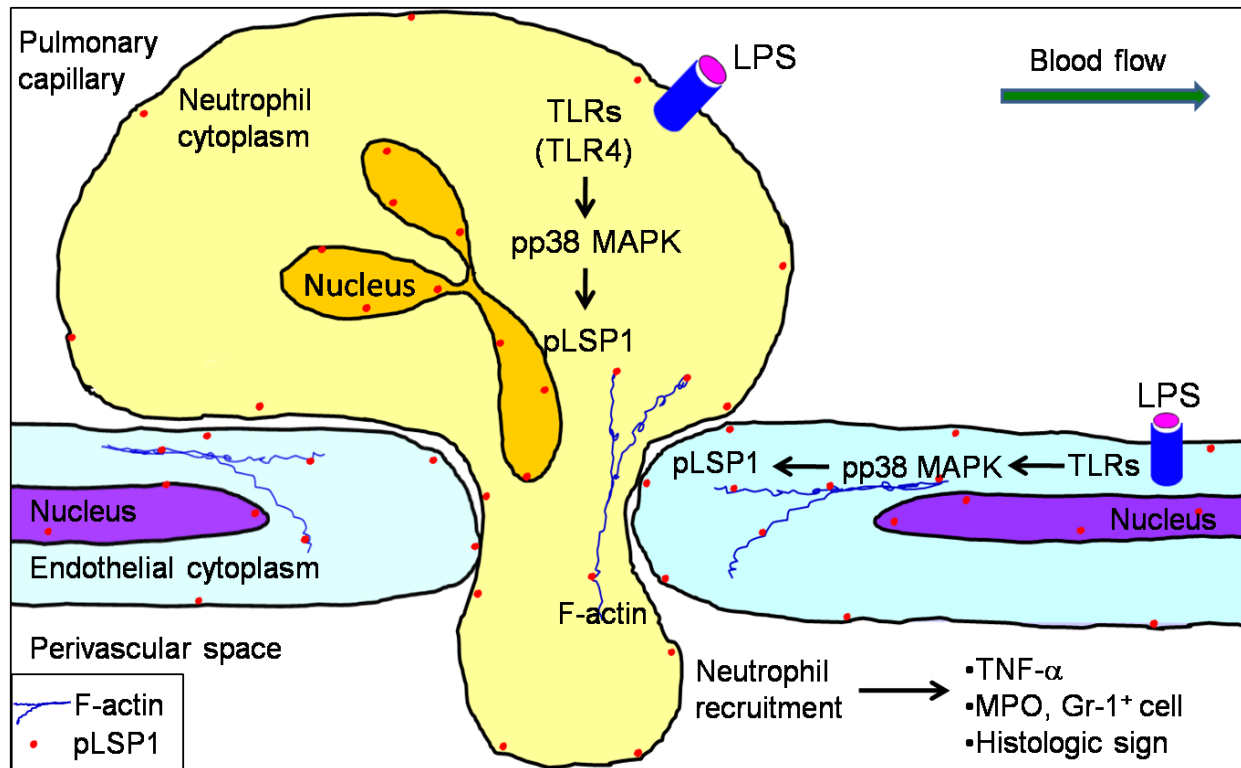


Figure 8.1. A schematic depicting LSP1 expression and its effects in acute lung inflammation

In addition to activate macrophage and epithelium in the airway, LPS can penetrate pulmonary capillary through an injured blood-air barrier (142) then binds to TLRs such as TLR4 molecule on neutrophils and endothelium (143, 144). It was proven before that LPS activates TLR4 which increases phosphorylation of p38MAPK (145, 146). Event downstream of p38MAPK activation is LSP1 phosphorylation which results in enhancing neutrophil migration. The host lung tissue is demolished due to an excessive extravasation of neutrophils and their products. MPO and TNF- α represent adverse effect mechanism on neutrophil recruitment and activation.

9. SUMMARY

In summary, the main purpose of this study was to determine the role of LSP1 in acute lung inflammation through its function in neutrophil migration and pathology in murine LPS-induced lung inflammation. We investigated that LSP1 expression is modulated in acute lung injury and LSP1 deficiency reduces neutrophil migration into LPS-induced acute lung inflammation in mouse model. Therefore, the proposed hypotheses are proven true. We expect that the results presumably apply to find a solution for lung inflammation. Inhibiting LSP1 could be one of the new therapeutic targets for acute lung inflammation involving LPS, an endotoxin in the Gram negative bacteria wall.

10. LIMITATION OF THE STUDY

These series of experiments only focus on studying LSP1 expression and its functions in an acute phase of lung inflammation which prominent neutrophil extravasation is one of the important hallmarks. Therefore, further studies are required to strengthen the results in future and to explore the impact of LSP1 on monocyte/macrophage recruitment in the later phase of acute lung inflammation.

11. FUTURE STUDIES

In my study, LPS-induced acute lung inflammation experiment has indicated clearly the function of LSP1 in neutrophil recruitment and its effects on severity of acute lung inflammation. In the future, it is necessary to study LSP1 role in later phases of lung inflammation revolving around some longer time-point LPS administrations to elucidate mechanistic endpoint. It would be interesting to first look at LSP1 function in other inflammatory cell populations such as macrophages, and lymphocytes in addition to neutrophils in inflamed lungs to elucidate the whole

effects of LSP1 in the all aspects of lung inflammation. The expression of LSP1 on macrophage also leads to a question whether this protein has a function on macrophage migration and phagocytosis of apoptotic neutrophils. Moreover, the similar vascular permeability level of wild-type and LSP1^{-/-} mice suggests that such studies will need to be conducted over longer time periods. A comprehensive study on the roles of LSP1 on other aspects of lung inflammation in different models such as asthma also needs to be studied.

Additionally, the immunohistochemistry results showing an increase expression of LSP1 in mouse lungs by 9 hours of LPS treatment raise a question if there are alterations in expression of LSP1 in other lung inflammation contexts such as an asthma situation. Moreover, the similar expression of LSP1 across human and mouse lungs further suggests that LSP1 probably functions similarly in acute lung inflammation in human and mouse in this model. Based upon LSP1 expression studied by immunohistochemistry and immune-gold electron microscopy method in this study, the next experiment should be identification the expression of LSP1 in normal and inflamed lungs of other animal species such as horse, cattle, pigs and chicken infected with several different pathogens. It is necessary to investigate the functions of LSP1 in pulmonary physiology and pathology in domestic animals, which could lead to new therapeutic approaches for lung diseases.

Lastly there is need to delineate the precise mechanism of LSP1 signaling pathway that effect neutrophil functions and apoptosis in respond to other pathogens under *in vitro* conditions. Studies of p38MAPK on normal and activated neutrophils isolated from wild-type and LSP1^{-/-} mice and the interaction of such neutrophils with isolated lung microvascular endothelium should be designed to strengthen the results. Moreover, combating microbes is one of important functions of neutrophils to protect the host in the pathogen in the early phase of infection (147).

In a previous study on proteomic analysis on human neutrophil granules, LSP1 was observed particularly in gelatinase granules and specific granules associating actin cytoskeleton activity and phagosomes (102). Therefore, examination of LSP1 and NETs association due to exocytosis of the granules in response to bacteria can be a potential experiment in future to indicate LSP1 functions. Moreover, neutrophils can clear the pathogen by their productions and ion movement, which also attract other immune cells to protect the body in next stage of immune system (56). However, overproduction can damage the host tissue (148). Recent works have shown a controversy of LSP1 functions on superoxide anion production of neutrophil under different treatments such as PMA and KC (10). Therefore, other set of questions is whether LSP1 plays a role in productions such as ROS by activated neutrophils in response to some pathogens. Finally, because LSP1 has been shown to have some conflicting association with apoptosis in B and T cells (112, 114), mechanistic data on the role of LSP1 in regulation of neutrophil apoptosis which prolongs lifespan of activated neutrophils is credited with increase in tissue pathology are required to be studied.

12. PRESENTATIONS AND AWARDS

- Poster presentation: L.N.P. Khanh, C. Shankaramurthy, L. Liu and B. Singh. “Leukocyte-Specific Protein 1 (LSP1) regulates neutrophil migration in acute lung inflammation”.
 - Presented at “WCVM Graduate Student Research Poster Days”, March 13-14th, 2013, Western College of Veterinary Medicine, University of Saskatchewan. ***Achieved 2nd place poster in Cell/Tissue pathobiology theme.***
 - Presented at “20th Annual Life & Health Sciences research day”, 15th March 2013, College of Medicine, University of Saskatchewan.
 - Presented at the Experimental Biology 2013 Annual Conference, in Boston, Massachusetts, MA 02210, USA, 20-24th April 2013.

- Poster presentation: L.N.P. Khanh, C. Shankaramurthy, L. Liu and B. Singh. “Role of Leukocyte Specific Protein 1 in acute lung inflammation”.
 - Presented at “5th Prairie Infectious Immunology Networking Conference”, 19th-21st September 2012. Hosted by “Infectious Immunology Research Interest Group & Immunology and Infectious Disease Research Group”, celebrated in Moose Jaw, Saskatchewan.
 - Presented at “Students’ poster competition”, 3rd October 2012. College of graduate studies and research, University of Saskatchewan.

- Poster presentation: L.N.P. Khanh, C. Shankaramurthy, L. Liu and B. Singh. “Role of Leukocyte Specific Protein 1 in neutrophil recruitment in lipopolysaccharide-induced lung inflammation”.
 - Presented at “WCVM Graduate Student Research Poster Days”, March 13-14, 2012, Western College of Veterinary Medicine, University of Saskatchewan.
 - Presented at “19th Annual Life & Health Sciences research day”, 9th March 2012, College of Medicine, University of Saskatchewan. ***Achieved 3rd place poster in Immunology theme.***

- Seminar “Role of Leukocyte-Specific Protein 1 in acute lung inflammation” in Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan on 14th November 2012.

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